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The consequences of sexual selection in the common wall lizard: insights following secondary contact and non-native introductions

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Declaration

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Statement of Co-Authorship

This thesis is based on the following manuscripts referred to in the text by their chapter number:

Chapter 2: While, G. M., S. Michaelides*, R. J. P. Heathcote*, **H. E. A. MacGregor***, N. Zajac, J. Beninde, P. Carazo, G. Pérez i de Lanuza, R. Sacchi, M. A. L. Zuffi, T. Horváthová, B. Fresnillo, U. Schulte, M. Veith, A. Hochkirch, and T. Uller. 2015. Sexual selection drives asymmetric introgression in wall lizards. Ecology Letters 18:1366-1375. (*** Joint Second Author**)

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A manuscript that is related to the work in Chapters 2-4 can be found in Appendix I:

Appendix I: Heathcote, R. J. P., G. M. While, **H. E. A. MacGregor**, J. Sciberras, C. Leroy, P. D'Ettorre, and T. Uller. 2016. Male behaviour drives assortative reproduction during the initial stage of secondary contact. Journal of Evolutionary Biology 29:1003–1015.

The following people and institutions contributed to the publication of work (or manuscripts in preparation for publication) undertaken as part of this thesis:

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Chapter 3: HEAM, TU and GMW conceived and designed the study. TU, GMW, HEAM, SM, PC, and GPL conducted field work. HEAM coordinated the enclosure experiment and collected behavioural data with the assistance of JB. HEAM, GPL, and PC collected data in the lab. HEAM and SM generated microsatellite data. HEAM analysed the data and wrote the manuscript with input from all authors.

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Often, all you see of a lizard is that retreating tail. But after a minute, an enquiring head appears, and another, wearing what I can't help but see as a smile. Or, as I approach more cautiously, a lizard cocks her head sideways to look up at me. They often do that.

Richard Kerridge

Cold Blood: Adventures with Reptiles and Amphibians

Abstract

Reproductive characters often vary geographically within species. This has led to the suggestion that traits related to reproduction evolve rapidly and that sexual selection is an important cause of diversification. Using the common wall lizard, *Podarcis muralis*, this thesis explores the consequences of interactions between reproductive characters and sexual selection in two environmental contexts: (i) following secondary contact between lineages that differ in secondary sexual characters, and (ii) following introduction to a cooler and more seasonal non-native environment. To address this, I adopted an integrated approach, combining information on associations between reproductive characters, social behaviour, and reproductive success in an experimental setting with extensive documentation of phenotypic variation across native and non-native populations.

Common wall lizards occupy a wide geographic range, spanning from western Spain to Turkey. Their phylogeographic structure is complex and composed of several genetically and phenotypically distinct lineages. In the first part of this thesis I examined how divergence in male sexual characters between two lineages – the Italian lineage, where males have highly exaggerated sexual traits, and the Western European lineage, where sexual traits in males are less expressed – mediates sexual selection and ultimately patterns of hybridization and introgression following secondary contact. Specifically, I combined an investigation of behavioural interactions and patterns of paternity in experimentally replicated mixed-lineage populations with genetic and phenotypic data from three independent zones of secondary contact. Experimentally, I show that Italian males have a significant advantage over Western European males in competition for females, leading to overall greater courtship and mating success, and consequently, asymmetric hybridization. Patterns of genetic and phenotypic introgression following secondary contact mirrored this directionality. Nuclear microsatellite markers revealed a westwards shift in the position of the hybrid cline compared to mitochondrial markers. Furthermore, clines in male visual sexual characters were shifted even further westwards into the Western European lineage, indicative of the rapid and adaptive displacement of Western European male sexual phenotypes. Combined with a lack of evidence for negative effects on hybrid offspring survival and their reproductive characters, these results demonstrate an important role for pre-copulatory sexual selection through male-male competition in shaping the genetic and phenotypic consequences of secondary contact.

I then examined the consistency of these effects across different communication channels, specifically comparing the above results for visual characters with chemical characteristics of male femoral secretions used as scent marks. Despite chemical communication being considered an important feature of lizard reproductive behaviour, I find little evidence for a role of divergence between the lineages in chemical characters in hybridization or sexually selected introgression. In contrast to the extensive introgression of the visual characteristics of the Italian lineage into the Western European lineage, patterns of introgression in chemical profiles resembled that of nuclear microsatellite markers, implying that genetic divergence in chemical characters is selectively neutral. These results highlight the potentially differing functions for visual and chemical communication channels in lizards. Chemical characters in wall lizards may function primarily as an individual-based recognition system.

In the second part of this thesis I examined divergence in female and male reproductive characters in response to a different climatic selection regime. Wall lizards that have been recently introduced into England (outside of their native distribution) experience a cooler, more seasonal climate that effectively restricts offspring recruitment to the first clutch of the season. This should exert strong directional selection on the reproductive investment of both females and males. Consistent with an adaptive response to climate, I show that non-native females in England produce relatively larger and heavier first seasonal clutches and smaller and lighter second seasonal clutches compared to native females. Despite non-native male fitness also depending almost entirely on the first clutch of the season, examination of male behaviour, dominance hierarchies, and phenotypic associations with mating and fertilization success in experimental populations revealed that non-native males do not alter their sexual strategies and compete aggressively to fertilize the second clutches from non-native females. These results highlight the potential for sex-specific limitations on rapid adaptive shifts in reproductive characters when male behaviour has been shaped by sexual selection regimes in past environments.

Combined, this thesis provides evidence of the potential ways in which sexual selection may shape the evolutionary and ecological trajectory of populations across different environmental contexts.

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Contents

Chapter 1

General Introduction

Sexual selection (Box 1) is a powerful evolutionary force. It can cause rapid diversification and exaggeration in male and female reproductive characters (e.g. Arnqvist 1998; Mead & Arnold 2004; Svensson & Gosden 2007; Shirangi *et al.* 2009), explain sex differences in life-history (e.g. Bonduriansky *et al.* 2008), and influence fundamental ecological and evolutionary processes (e.g. speciation, Boughman 2001; extinction, Doherty *et al.* 2003; rates of molecular evolution, Dorus *et al.* 2004; Petrie & Roberts 2007). Given the compelling and sometimes bizarre phenotypic diversity that can be attributed to sexual selection it is no surprise that the topic has been researched intensively from both a theoretical and empirical perspective (reviewed, Andersson 1994; Ritchie 2007; Cornwallis & Uller 2010; Kuijper *et al.* 2012; Miller & Svensson 2014). For example, mathematical models have outlined how female preferences for male traits can evolve (e.g. Lande 1981; Grafen 1990; Kirkpatrick & Hall 2004), the experimental removal of sexual selection has demonstrated dramatic phenotypic consequences in model organisms (e.g. Pitnick *et al.* 2001), field studies are revealing the temporal and spatial dynamics of sexual selection in the wild (e.g. Clutton-Brock *et al.* 1997; Gosden & Svensson 2008), and genomic data are increasingly providing opportunities to characterize the genetic mechanisms and signatures of sexual selection (Wilkinson *et al.* 2015).

Despite the substantial evidence for the ecological and evolutionary consequences of sexual selection, it is only relatively recently that the context-dependent nature and effects of sexual selection (Figure 1.1) have received significant empirical attention (Cornwallis & Uller 2010; Miller & Svensson 2014). Current interest has been motivated, in particular, by evidence for temporal fluctuations in sexual selection within populations in a variety of taxa (e.g. Olsson *et al.* 2011; Robinson *et al.* 2012; Wacker *et al.* 2014), and the possibility that population-level

divergence in sexually-selected traits as organisms adapt to new social and ecological conditions may provide a link between micro-evolutionary processes and macro-evolutionary patterns (West-Eberhard 1983; Panhuis *et al*. 2001). For example, theoretical models and empirical data suggest that ecological selection and sexual selection may often interact during the formation of new species (van Doorn *et al.* 2009; Maan & Seehausen 2011). Despite this increased focus on environmental context, we still have only a limited understanding of how and why patterns of sexual selection vary in space and time (Hoekstra *et al.* 2001; Kingsolver *et al.* 2001; Jones & Ratterman 2009). As a result, it remains a major challenge to predict the implications of different sexual selection regimes for both the direction and tempo of evolution as well as population and community dynamics. To address this, studies in a broader range of taxa and across a wider range of environmental conditions are needed. To contribute towards this topic, this thesis focuses on two specific environmental contexts where the mechanisms, strength and targets of sexual selection can have significant consequences at the population-level: (i) when lineages come into secondary contact, and (ii) when species are introduced to environments that differ from those previously encountered.

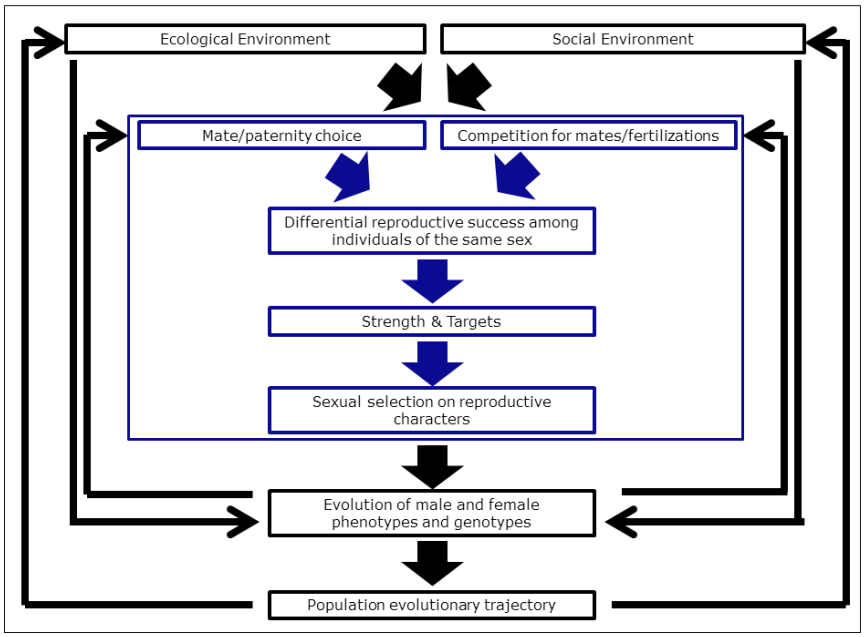

Figure 1.1: Conceptual diagram to illustrate that the nature and effects of sexual selection (blue box) depend on the environmental context.

Box 1: **Basic ideas of sexual selection**

Definition. Sexual selection describes two mechanisms: intra-sexual competition for mates and fertilizations (typically among males), and inter-sexual mate choice decisions (typically by females) that generate variance in reproductive success among individuals of the same sex (Darwin 1859; Darwin 1871; reviewed by Andersson 1994). More formally, sexual selection can be defined as a functional relationship (linear, disruptive, or stabilizing in form) between the phenotypic value of a heritable trait and relative reproductive fitness through the independent or combined actions of mating competition and mate choice. The higher the covariance between a trait and fitness via these mechanisms, the greater the intensity of sexual selection, and the stronger the expected evolutionary response from one generation to the next (assuming expressed heritable genetic variation in a character under sexual selection, see Merila *et al.* 2001).

Compete or choose? Theoretically, the sex that makes the smaller investment in terms of cost to their future reproduction and has the higher potential reproductive rate will experience stronger sexual selection and be subjected to both intra- and inter-sexual selection (Bateman 1948; Trivers 1972; Shuster & Wade 2003). Therefore, because males make the smaller per gamete investment, in most species, they are the competing sex, and express behaviours and suites of correlated morphological and physiological traits (secondary sexual characters) that function in physical combat, or as communication channels to signal competitive ability or attract females (Janicke *et al.* 2016). Furthermore, because females often mate multiply during an episode of reproduction, sexual selection can extend beyond copulation (Pizzari *et al.* 2002). Post-copulatory sexual selection on males can drive evolutionary changes in primary (e.g. testes mass, sperm number, sperm morphology) and secondary (e.g. mate guarding, copulatory plugs) sexual characters that increase fertilization opportunities or success under sperm competition. From a female perspective, pre- and post-copulatory sexual selection favours the behavioural and physiological ability to discriminate among competing males and gametes. Generally, females are assumed to seek mates that either maximise direct benefits (e.g. nuptial gifts, territory and parental care, Trivers 1972) or offspring genetic quality or attractiveness (Fisher 1915, 1930).

Trade-offs. Sexual selection drives the evolution of traits that are well adapted to the intraspecific social environment (or more specifically the sexual environment) and achieving mating success. Hence, the expression of secondary sexual characters typically comes at a cost to other components of fitness (e.g. Zuk & Kolluru 1998). This is especially evident for sexually selected traits that directly serve an individual in both mating and non-mating contexts (e.g. Bro-Jorgensen et al. 2007). Here, sexual and non-sexual fitness components are subjected to a trade-off (e.g. Robinson et al. 2006), and directional sexual selection is expected to be counteracted by other selective forces acting in opposing directions. In stable environments sexual phenotypes are generally assumed to represent an evolutionary balance between mating success, survival, and fecundity (Kokko & Brooks 2003). However, changes to social or ecological conditions may impose new costs and benefits on sexual characters leading to trait divergence or convergence among populations, which in turn can influence evolutionary diversification.

1.1 The diverse outcomes of secondary contact

Populations, lineages and species naturally undergo periods of relative isolation and divergence followed by contact (secondary contact), which occurs at different spatial scales. For instance, the glacial-interglacial oscillations of the Pleistocene caused cycles of species' range contractions and expansions across the northern and southern hemispheres (Taberlet *et al.* 1998; Hewitt 2004; Schmitt 2007). As a result, many extant animals and plants have come into natural secondary contact following the last glacial maximum (~ 18,000 years ago) (e.g. Taberlet *et al.* 1998). In contrast, dispersal of a few individuals across geographic barriers can result in highly localised instances of secondary contact (e.g. between island endemic honeyeaters, Sardell & Uy 2016). While secondary contact between divergent lineages occurs as a natural phenomenon, human activities remove geographic barriers between taxa through the intentional or accidental translocation of organisms and habitat disturbance (e.g. Bleeker & Hurka 2001; Michaelides *et al.* 2015). Current rates of secondary contact are predicted to increase further during the Anthropocene as species ranges shift to cope with global climate warming (e.g. Garroway *et al.* 2010). This highlights the need for an improved understanding of the potential outcomes and significance of secondary contact for biodiversity (Scriber 2014).

The dynamics of ecological and evolutionary processes in regions of secondary contact are fascinating because they have a profound impact on the fate of evolutionary lineages (e.g. Taylor *et al.* 2006) and the structure of ecological communities (e.g. Whitham *et al.* 1994). For the lineages that meet, several broad outcomes are possible reflecting how they have diverged in isolation, the nature of reproductive interactions, and the extent and direction of hybridization. Firstly, individuals from parental lineages may not interbreed due to behavioural differences, or mechanical or genetic incompatibility (i.e. speciation is complete and reproductive barriers are impermeable). Even in the absence of hybridization, interactions, in particular mating interactions, between reproductively isolated lineages, can act as a potent source of selection on

reproductive characters (e.g. Amézquita *et al.* 2006; Drury *et al.* 2015). However, unless divergence has caused complete pre- or post-zygotic reproductive isolation, secondary contact will be followed by hybridization with several potential evolutionary outcomes. Hybridization can promote the evolution of pre-zygotic reproductive barriers via character displacement and reinforcement if there are fitness costs in either lineage (Dobzhansky 1937; Ortiz-Barrientos *et al.* 2009); cause the extinction of a rare lineage (Rhymer & Simberloff 1996; Todesco *et al.* 2016); increase biodiversity with the formations of reproductively isolated hybrid lineages (i.e. hybrid speciation, Mallet 2007; Salazar *et al.* 2010); or, as long as some fertile hybrids are formed, facilitate introgression, the movement of genetic material from one lineage to another (Anderson & Hubricht 1938; Anderson 1949; Harrison 1993). Introgression is emerging as a potent evolutionary force that can drive the tempo and direction of evolution in hybridizing taxa. Since a large proportion of the world's biodiversity is estimated to be of relatively recent evolutionary origin (< 5 million years), susceptibility to hybridization and introgression following secondary contact may be more common than is recognized (Seehausen et al. 2008).

The nature and extent of introgression has been shown to vary substantially across interacting lineages and depending on the genomic regions involved. For example, between lineages, introgression may be extensive (e.g. Melo-Ferreira *et al.* 2005) or restricted to a narrow zone of contact (e.g. Singhal & Moritz 2012), bi-directional (e.g. Sequeira *et al.* 2005) or asymmetric (e.g. Baldassarre *et al.* 2014), transient (e.g. Dasmahapatra *et al.* 2002) or persistent over many generations (e.g. Alexandrino *et al.* 2005), and neutral or adaptive (Box 2). At the genomic-level, the counteracting forces of divergent selection and gene flow can lead to a mosaic of genomic regions experiencing either divergence or homogenization (e.g. Scascitelli *et al.* 2010; Teeter *et al.* 2010). By studying these patterns, we can gain insights into the traits that may contribute to reproductive isolation, are selectively neutral, or confer fitness benefits in the receiving lineage. For instance, Toews et al. (2016) recently demonstrated that the genomes of hybridizing blue- and golden-winged warblers are largely homogenous due to episodes of introgression, but the

authors also identified small divergent genomic regions associated with plumage-colour differences between the species.

Box 2: Adaptive introgression

The question of whether hybridization and introgression have largely positive or negative implications for adaptation has been one of historical controversy. Early theoretical models predicted that introgression should disrupt local adaptation (Haldane 1930; Balkau & Feldman 1973). This idea, combined with the assumption that hybridization was rare (Mayr 1942), as well as a conceptually incompatible with the biological species concept (Mayr 1963), resulted in an underappreciated role of introgression in animal evolution. Nonetheless, introgressive hybridization has been a topic of plant science research for decades (Anderson 1949; Anderson & Stebbins 1954), which has inspired current interest in understanding the evolutionary significance of introgression in animals outside of a speciation framework (e.g. Hedrick 2013; Rius & Darling 2014). In addition to the fact that introgression may augment genetic variation in receiving lineages (potentially increasing their future adaptive potential), it is now widely accepted that introgression itself can be adaptive. Adaptive introgression refers to the transfer of specific alleles or allele combinations that confer a survival, fecundity or mating advantage in the genetic background and environment of the recipient population. Importantly, introgression can be a highly effective source of adaptation because adaptive traits that would otherwise require multiple *de novo* mutations or alleles can be acquired directly for another lineage (Rieseberg 2009). Introgression favoured by selection can be extensive despite negative fitness consequences for hybrid offspring i.e. even when hybridization is not adaptive (Arnold *et al.* 1999). Until recently there have been few convincing examples of adaptive introgression in animals. Demonstrating adaptive introgression is challenging because multiple lines of evidence are required, including, the characterization of the genetic basis of a trait, evidence for significant positive fitness effects of the trait in the genetic background or environment of the recipient lineage, and an evolutionary history to confirm that the trait did not arise through incomplete lineage sorting (Tigano & Friesen 2016). However, with the advent of accessible genetic and genomic tools, cases of adaptive or putative adaptive introgression are increasing in number. These encompass a broad range of taxa with most studies identifying viability as the selective agent acting on introgressing traits (Table 1.1).

Table 1.1. Recent studies documenting adaptive or putative adaptive introgression in animals.

Donor taxon	Recipient taxon	Trait	Benefit/suggested benefit	Selection	References
Algerian mouse (*Mus spretus*)	House mouse (*Mus musculus domesticus*)	Pesticide resistance	Survival against pesticides	Survival	(Song *et al.* 2011; Liu *et al.* 2015)
African malarial mosquito *(Anopheles gambiae)*	African malarial mosquito *(Anopheles coluzzii)*	Pesticide resistance	Survival against pesticides	Survival	(Norris *et al.* 2015)
Domestic dog (*Canis lupus familiaris*)	North American Wolf (*Canis lupus subsp)*	Black coat colour	Improved camouflage in forest habitat	Survival	(Anderson *et al.* 2009)
Heliconius butterflies *(Heliconius sp)*	Heliconius butterflies *(Heliconius sp)*	Wing colour pattern	Müllerian mimicry	Survival	(Heliconius Genome Consortium 2012; Pardo-Diaz *et al.* 2012)
Darwin's finches *(Geospiza sp)*	Darwin's finches *(Geospiza sp)*	Beak shape	Access to food resources	Survival	(Lamichhaney *et al.* 2015)
Denovisians *(Denisova hominin)*	Humans from Tibet (*Homo sapiens*)	Response to hypoxia	Adaptation to high altitude	Survival	(Huerta-Sanchez *et al.* 2014)
Domestic goat *(Capra aegagrus hircus)*	Alpine ibex *(Capra ibex ibex)*	MHC allele	Enhanced immune response	Survival	(Grossen *et al.* 2014)
White-collared manakin (*Manacus candei)*	Golden-collard manakin *(Manacus vitellinus)*	Golden plumage	Female preference in leks	Mating	(Stein & Uy 2006)
Red-backed fairy wren (*Malurus melanocephalus cruentatus*)	Orange-backed fairy wren (*Malurus melanocephalus melanocephalus*)	Red plumage	Advantageous in extra-pair mating	Mating	(Baldassarre & Webster 2013; Baldassarre *et al.* 2014)

1.1.1 The role of sexual selection in hybridization and introgression

Since hybridization in animals is a behavioural phenomenon, the question of what causes some individuals hybridize can be approached in the context of mating systems and sexual selection (Willis 2013). The nature of divergence in reproductive characters between lineages in allopatry will be especially important in this context, affecting the strength, direction and targets of sexual selection within and between the lineages upon secondary contact, with implications for patterns of hybridization (e.g. Rosenfield & Kodric-Brown 2003; Baldassarre & Webster 2013). Sexual selection (or more broadly, social selection, *sensu* Crook 1972) differs from other forms of natural selection in that the fitness implications for a focal individual depend not only on their phenotype but also on the phenotypes of the individuals with whom they interact i.e. the phenotypic composition of the social environment (Wolf *et al.* 1999; Lyon & Montgomerie 2012). Thus, the traits that mediate male-male competition and female choice are both the targets and the agents of selection. Considering that secondary contact often brings together phenotypically divergent individuals, potential mates and competitors may vary considerably more in their expression of sexually selected characters following secondary contact than within allopatric populations of either lineage, and this can be the cause of hybridization. For example, if females of two lineages have diverged in body size, and males of both lineages prefer larger females (due to associations with higher fecundity), asymmetric hybridization may occur (Schmeller *et al.* 2005).

The phenotypic targets of sexual selection in regions of secondary contact can become the phenotypic targets for introgression and thereby shape the dynamics of hybrid zones. Associations between divergence in sexual selected characters, hybridization, and the strength of introgression have been documented in a number of systems. For instance, divergent male aggression and female visual preferences for male mating colours interact to promote hybridization between Pecos pupfish and sheepshead minnow, resulting in a hybrid swarm

(Rosenfield & Kodric-Brown 2003). In contrast, divergent ecological selection on warning colour patterns that also function in male mate choice promotes assortative mating, and limits introgression between species of *Heliconius* butterflies (Merrill *et al.* 2014). Several studies have also identified a link between the targets of sexual selection upon secondary contact and phenotypic patterns of introgression in those same traits, suggestive of adaptive introgression (see Box 2). For instance, extra-pair mating behaviour in concert with female preferences for red plumage colour drives asymmetric hybridization and the introgression of red plumage between orange-backed and red-backed subspecies of fairy wrens (Baldassarre & Webster 2013). In Central America, golden-collared manikins and white-collared manikins hybridize asymmetrically because females prefer golden-collard males, which results in the spread of golden plumage colour (Stein & Uy 2006). These examples represent most of our evidence for adaptive introgression in the context of sexual selection to date. This is perhaps partly because the strength, direction and targets of sexual selection upon secondary contact are often insufficiently understood. Alternatively, it could be that divergent sexually selected characters are often under diversifying rather than directional selection in regions of secondary contact, and hence function more as contributors to reproductive isolation than as targets for introgression. To distinguish between these possibilities, it will be useful to study young regions of secondary contact because in these areas the processes that promote introgression or reproductive isolation may be most easy to discern.

1.2 Sexual selection in the context of ecological change

Another context where changes in environmental conditions are expected to impact on patterns of sexual selection is when organisms are exposed to novel ecological environments. This can occur through natural or human-mediated dispersal or through ongoing environmental change. New ecological conditions may interact directly or indirectly with the mechanisms, strength and targets of sexual selection. Most evidence for the effects of ecological factors on sexual selection falls within one of four broad categories: (i) the demographic effects of breeding resource availability and its implications for intensity of mating competition; (ii) altered natural selection regimes and associated changes in the relative costs of sexual traits; (iii) the effects of nutrient availability on the expression of condition-dependent sexual traits; and (iv) environmental effects on the transmission efficiency of sexual signals and its implications for mate choice and competition. For example, an environment with highly concentrated breeding resources intensifies directional selection on male gonad size and dominance signals in the European bitterling, *Rhodeus amarus* (Reichard *et al.* 2009). In a classic example of rapid and adaptive micro-evolutionary change, the intensification of predation regimes causes a reduction in the expression of body colouration selected through female choice in male Trinidadian guppies, *Poecilia reticulata* (Endler 1980). Experimental food restriction reduces the opportunity for sexual selection and increases the variance contribution of post- compared to pre-copulatory sexual selection in freshwater snails, *Physa acuta* (Janicke *et al.* 2015). Eutrophication induces plastic changes in the male sexual behaviour which counteracts the reduced transmission efficiency of visual cues important for mate attraction in sticklebacks, *Gasterosteus aculeatus* (Engström-Öst & Candolin 2007). These examples demonstrate that sexually selected characters may respond to new ecological conditions in highly complex ways influenced by the interacting effects of new sexual selection regimes, natural selection, and plastic responses to conditions. Thus, the implications of sexual selection and sexual characters for individual

fitness, and for the trajectories of populations in changing environments, are often difficult to predict.

Studying associations between temporal ecological change, reproductive characters, and population persistence in the wild is usually limited by the availability of long-term data. However, the study of populations occupying a newly colonized environment or having been recently introduced from a common ancestral source offers an alternative approach. These populations can reveal the capacity of organisms to adapt across a range of ecological contexts and the factors that contribute to divergence in sexually selected characters. For example, the sexually selected characters of introduced Trinidadian guppies in northern Australia have diverged rapidly among populations, and the strength and direction of sexual selection was found to explain most of the variation at the population-level (Lindholm *et al.* 2014). Dark-eyed juncos, *Junco hyemalis,* that have colonized less seasonal climates on the Pacific coast of North America, have diverged from ancestral populations in tail plumage, a sexually selected signal. However, in contrast to guppies, this has been attributed to the effects of the trait on juvenile mortality in late season clutches and not changes to the environmental context of sexual selection (e.g. Yeh 2004; Price *et al.* 2008). More generally, identifying the selective pressures, whether linked to sexual selection, or survival at the adult or offspring stage, is critical for establishing if sexual phenotypes are adaptive in new environments.

1.2.1 Invasions, life-history, and sexual strategies, and their implications for population viability

Social and ecological factors can have direct and indirect effects on the expression of traits that are related reproduction, including sexually selected characters. Given the causal link between reproductive characters and fitness (either directly through fecundity and mating success, or indirectly due to trade-offs among fitness components), successful colonization often requires responses that maintain successful reproduction under new conditions. To date, our understanding of adaptive or evolutionary shifts in reproductive strategies during invasions has largely been based on studies of female reproductive effort (particularly in non-native fish, e.g. Haynes & Cashner 1995; Novomeska & Kovac 2009; Masson *et al.* 2016). In contrast, very little is known about how male sexual strategies and life-history respond during the invasion process, and the consequences for male-male competition and the intensity of sexual selection (see Laugier *et al.* 2013 for a recent study addressing a similar theme).

Males and females typically differ in their reproductive strategies because female fitness varies most as a function of fecundity selection while male fitness often varies as a function of sexual selection. The poor timing of allocation to reproduction in new environments is predicted to have more severe consequences for the life-time reproductive success of females than for males due to their greater per gamete investment in reproduction. Nonetheless, male sexual behaviour also comes with potential costs to males and also to females, including risk of sexually transmitted disease, predation, decreased lifespan and risk of female harm (Daly 1978). Therefore, male investment in characters that do not contribute to successful reproduction could reduce the overall life-time reproductive success of both sexes. At a more mechanistic level, since the two sexes have evolved to coordinate their reproduction, responses in one sex, whether plastic or genetic, should shape the ability for responses in the other. Females are predicted to alter their reproductive strategies in response to environmental cues such as temperature and nest site quality that directly impact on offspring survival (Ball & Ketterson

2008). Thus, reproductive responses in males, particularly in species without parental care, may depend more on how females alter their reproductive life-history than the direct effects of the environment.

From an ecological perspective, the ability of males to modify their sexual strategies to coordinate with environmental conditions and the changing circumstances of female reproduction may affect reproductive output at the population-level, and hence have implications for the persistence and growth of invasive populations. Ultimately, the influence of sexual selection on population viability under new or changed conditions will depend on two key factors. First, it will depend on how past environments have shaped the underlying genetic variation and plasticity in sexually selected characters. For example, strong directional selection can deplete standing genetic variation in traits, reducing their adaptive potential under new selection regimes, and this could be the case for many characters under sexual selection (Hoekstra et al. 2001; although see Kotiaho *et al.* 2008). In general, rapid micro-evolutionary responses are often limited by the degree and nature of genetic diversity, particularly if the mismatch between past and present conditions is large. As a result, plasticity, especially behavioural plasticity, has attracted particular attention as a mechanism for rapid and adaptive responses to environmental change, lessening extinction risk, and allowing time, facilitating, or even removing the need for adaptive micro-evolutionary change (Price *et al*. 2003; West-Eberhard 2003). However, even if plasticity in sexually selected characters has evolved (e.g. Cornwallis & Birkhead 2008), perhaps in response to environmental variability in past environments, there is no guarantee that plastic responses will be adaptive under new conditions (Greenfield & Rodriguez 2004). Second, the influence of sexual selection will depend on how sexual characters affect components of fitness (survival, fecundity, mating success), and, thus, offspring number and quality in new environments. One way in which sexual selection could have negative fitness consequences at the population-level is if either low quality males or males that are poorly adapted to the prevailing environment achieve the highest mating

success. For example, when sexual communication is impeded by environmental conditions or when the honesty of condition-dependent signals is broken down. However, linking evidence of negative fitness effects of sexual selection or sexually selected traits at an individual-level to population mean fitness and population dynamics remains challenging. Some theoretical work suggests that strong sexual selection may increase the probability of extinction and population decline during periods of environmental change because sexual selection typically shifts characters away from their survival optima (e.g. Tanaka 1996; Houle & Kondrashov 2002; Kokko & Brooks 2003). In contrast, models have also suggested that the negative demographic consequences of sexual selection may be self-limiting if reductions in population density also correspond to a reduction in the intensity of sexual selection (e.g. Rankin 2007). Furthermore, sexual selection contributes to the purging of deleterious mutations (Whitlock & Agrawal 2009), which should improve population mean fitness and facilitate rapid adaptation. However, experimental evolution studies suggest that sex-specific adaptation can be impeded by sexual conflict (e.g. Delcourt *et al.* 2009; Chenoweth *et al.* 2015). Comparative studies of introduced species have similarly produced mixed results, with evidence suggesting strong sexual selection increases extinction risk (e.g. Sorci *et al.* 1998) or has limited effects (e.g. Cassey *et al.* 2004). A crucial first step towards understanding the population-level consequences of sexual selection and sexually selected traits under novel conditions is to establish how reproductive characters mediate sexual selection in new environments and the extent to which they respond adaptively to changes in conditions.

1.3 The common wall lizard as a study system

In this thesis I use the common wall lizard, *Podarcis muralis* (Laurenti 1768), as a study system to examine the role of sexual selection following secondary contact and the response of sexual selected characters following introduction to a new climate.

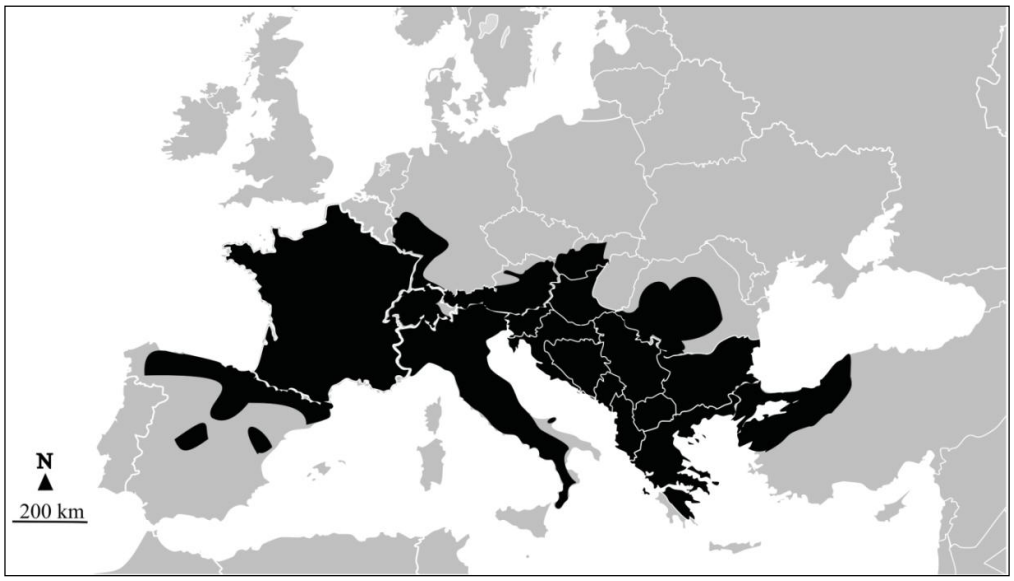

Figure 1.2: Map to show the native geographic range of *Podarcis muralis* (black).

P. muralis has a geographic range spanning from western Spain to Turkey (Figure 1.2). Across their native range, phylogeographic structure is pronounced with at least eight major genetic lineages described based on mitochondrial haplotype data (Schulte *et al.* 2012a). Furthermore, phenotypic distinctions among several lineages have led to their description as separate subspecies (Böhme 1986). It is generally assumed that periods of allopatric isolation among populations at different points during the Pleistocene climatic oscillations promoted the intraspecific genetic and phenotypic diversity observed today (Giovannotti *et al.* 2010; Salvi *et al.* 2013). Two lineages, found on the Italian peninsula, are the focus of investigations in this thesis – the Western European lineage (ranging from eastern France to the Southern Alps) and the Italian lineage (specially the Tuscan and Venetian mitochondrial clades that are native to north-central Italy).

1.3.1 Secondary contact and hybridization

The Western European and Italian lineages of wall lizards have come into secondary contact in a number of different contexts. After the last glacial maximum on the Italian peninsula, for example, the Italian lineage, which resided in southern glacial refugia, expanded its range northwards leading to secondary contact with the Western European lineage in north-central Italy. However, the extent and direction of hybridization and gene flow between the lineages is unknown. More recent zones of secondary contact between these lineages also exist as a result of non-native introductions. For example, in Holmsley, England, both lineages were introduced in the 1980's (Michaelides *et al.* 2013), and in the Manheim region in western Germany Italian individuals were introduced into Western European populations (Schulte *et al.* 2012c).

There is an overwhelming focus on pre-copulatory female choice in the context of sexual selection and hybridization, with relatively little attention given to the role of male-male competition (which mirrors a research bias towards the study of female choice in general, McCullough *et al.* 2016). Wall lizards are an excellent model to study the contribution of male-male competition to hybridization and introgression. Firstly, common wall lizards are sexually dimorphic, as is typical of the lacertid group (Braña 1996), however, the degree of sexual dimorphism varies geographically (e.g. Aleksić et al. 2009), including between the Western European lineage, where sexual dimorphism is weak, and Italian lineage, where sexual dimorphism is strong. Secondly, there is limited evidence for pre-copulatory mate choice in lizards (Olsson & Madsen 1995; Tokarz 1995) suggesting that variation in the intensity of pre-copulatory male-male competition has been the primary driving force in the origin and, for some populations, exaggeration of male secondary sexual characters (Figure 1.3).

Figure 1.3: Images of male *P. muralis* to show (a) differences in dorsal body colouration between the Italian lineage (green-backed) and the Western European lineage (brown-backed), (b) outer-ventral scale UV-blue ornamentation, (c) bite-force testing of an Italian male, (d) the femoral pores of a male during the breeding season with secretions visible.

In the first part of this thesis I address the consequences of divergence in reproductive characters between the Italian and Western European lineages for the strength and targets of sexual selection via male-male competition, and its implications for patterns of hybridization and introgression following secondary contact.

1.3.2 Non-native introductions

Wall lizards have been successfully introduced to parts of Germany (Schulte 2008; Schulte *et al.* 2012a, b, c), North America (Allan et al. 2006), and England (Michaelides et al. 2013; Michaelides et al. 2015) over the last century. Colonization of *P. muralis* in England has been facilitated by both private collectors and the pet-trade, involving at least nine introduction events from multiple native sources (Michaelides *et al.* 2015). At least 10 historically documented

populations are known to have gone extinct (Langham 2016). The majority of the 23 extant populations are found along the south coast of England (Figure 1.4) and comprise of individuals of the Italian lineage (Michaelides *et al.* 2013).

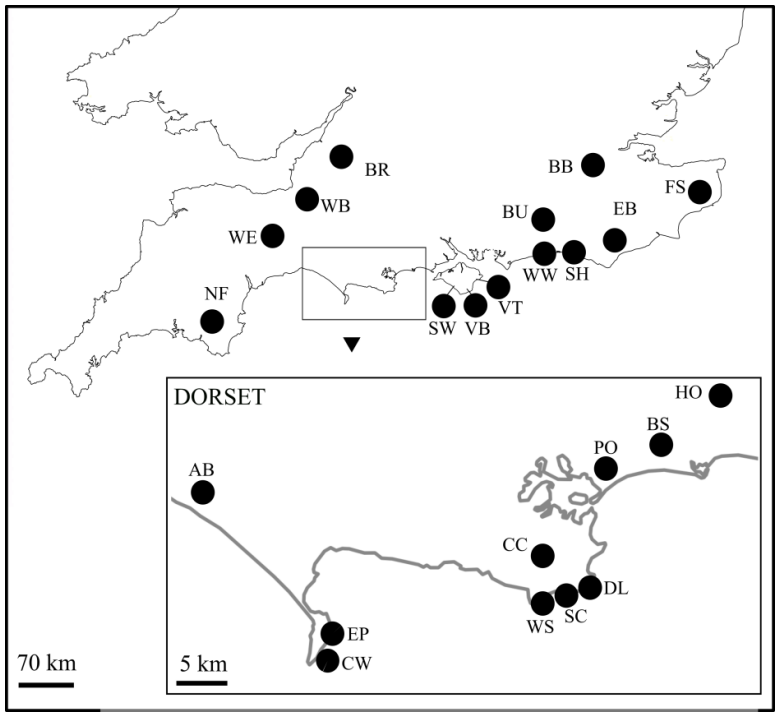

Figure 1.4: Map of the 23 extant non-native populations of *P. muralis* in England (adapted from Michaelides *et al.* 2013).

Climate matching is a factor which was recently identified as a key predictor of establishment success in non-native reptiles (Mahoney *et al.* 2015). Spring-summer temperatures are around 10 degrees cooler in the United Kingdom compared to Italy. Consequently, the long-term survival of introduced populations in England may depend on the ability of individuals to adapt to these cool summer temperatures. While adaptive responses in female reproductive physiology have been recently documented in non-native populations (While *et al.* 2015), it remains unclear whether female and male reproductive strategies have also shifted in response to a new climatic regime and strong seasonal constraints on the timing of offspring recruitment. Clear predictions can be made regarding optimal reproductive investment strategies in England. Specifically because second clutches make little contributions to the life-time reproductive

success of introduced lizards, selection should favour individuals who invest more in the first clutch of the breeding season. Hence, this system is a useful model to examine the adaptive potential of life-history characters and sexually selected behaviour following a recent and sudden change in climatic selection regime. The second part of this thesis examines how female life-history characters and sexually selected behaviour have diverged between native and non-native populations by integrating observations in experimental populations with phenotypic data from natural and introduced Italian-origin populations.

1.4 Thesis outline

The main body of this thesis is four data chapters formatted for submission in peer-reviewed journals. Each chapter can be read independently, however, **Chapter 4** relies on methods described in **Chapters 2** and **3**.

In **Chapter 2** experiments are used to generate predictions regarding the strength and direction of gene flow between the Italian and Western European lineages, by examining the role of sexual selection during initial secondary contact, and the fitness consequences of hybridization. Three zones of secondary contact, including one natural hybrid zone and two contemporary instances of contact, are used to test these predictions. Patterns of genetic and phenotypic introgression are examined using cline analyses and the experimental and cline data are discussed in the context of sexually selected introgression.

The phenotypes of hybridizing males and the contribution of hybridization to variance in male reproductive success are examined in **Chapter 3.** Here, the consequences of divergence in male visual and behavioural sexual characters for the strength and targets of sexual selection and the extent and direction of hybridization upon secondary contact are investigated in an experimental setting. Some putative phenotypic targets for ongoing sexual selection are identified in both lineages. Evidence for differences in the phenotypes of males gaining within-lineage compared to between-lineage fertilization success are considered within the context of known patterns of phenotypic introgression described in **Chapter 2**.

The influence of chemical communication on patterns of hybridization and introgression is investigated in **Chapter 4**. Differences in chemical characters between the lineages are described and the role of chemical divergence for spatial organisation, behaviour, and reproductive success is examined in experimental mixed-lineage populations. Predictions regarding patterns of chemical introgression are tested in a natural hybrid zone and the results

are compared with **Chapter 2**. Possible explanations for the contrasting patterns of neutral introgression in chemical characters and selective introgression in visual characters are discussed.

The consequences of introduction to a non-native environment for female and male reproductive investment are investigated in **Chapter 5.** A combination of field data and experiments in outdoor enclosures are used to test whether females in non-native populations increase their relative investment in their first clutch and lower investment in their second clutch compared to native females, and whether non-native males show reduced sexual competition following the first reproductive bout compared to native males. Associated divergence in body size and sexually selected traits between native and non-native populations is also investigated.

In **Chapter 6**, the general discussion, I summarise and discuss the main findings.

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Chapter 2

Sexual selection drives asymmetric introgression in wall lizards

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2.1 Abstract

Hybridization is increasingly recognized as an important cause of diversification and adaptation. Here we show how divergence in male secondary sexual characters between two lineages of the common wall lizard (*Podarcis muralis*) gives rise to strong asymmetries in male competitive ability and mating success, resulting in asymmetric hybridization upon secondary contact. Combined with no negative effects of hybridization on survival or reproductive characters in F1-hybrids, these results suggest that introgression should be asymmetric, resulting in the displacement of sexual characters of the sub-dominant lineage. This prediction was confirmed in two types of secondary contact; across a natural contact zone and in two introduced populations. Our study illustrates how divergence in sexually selected traits via male competition can determine the direction and extent of introgression, contributing to geographic patterns of genetic and phenotypic diversity.

Keywords: Introgression, Hybridization, Male-Male Competition, Female Choice, Lizards

2.2 Introduction

Gene transfer between species, sub-species or genetic lineages via hybridization is increasingly recognized as an important cause of diversification and adaptation (Barton 2001; Arnold 2007; Soltis & Soltis 2009; Abbott *et al.* 2013; Hedrick 2013). Because hybridization does not necessarily lead to an even mix of genetic and phenotypic characters of the parental lineages, it can cause new characters to arise or existing characters to be unequally transferred between lineages. This may be particularly likely when phenotypes that have diverged in allopatry confer a fitness advantage to one lineage upon secondary contact, making it advantageous for the other lineage to express the same characters. For example, expression of hetero-specific characters can have a survival advantage, which has been suggested to explain introgression of wing patterns between *Heliconius* butterflies (Pardo-Diaz *et al.* 2012), pest resistance in mice (Song *et al.* 2011) and the evolution of climate adaptation and herbivore resistance in sunflowers (Whitney *et al.* 2006, 2010). Alternatively, characters that confer a reproductive advantage in the competition for mates can enhance hybridization rates as well as provide hybrids with a selective advantage relative to subdominant pure-bred competitors. In the absence of severe genetic incompatibilities, this may enable secondary sexual characters to rapidly spread from one lineage to another (Prado *et al.* 2009; Parsons *et al.* 1993; Baldassarre *et al.* 2014).

Sexually selected hybridization has primarily been studied with respect to female choice. While female choice will often restrict gene flow (e.g., Saetre *et al.* 1997; Seehausen *et al.* 2008), increasing evidence suggests that it can also lead to asymmetric rates of hybridization and introgression of male sexual characters (McMillan *et al.* 1997; Wirtz 1999a; Stein & Uy 2006; Pfennig 2007; van der Sluijs *et al.* 2008). For example, in a hybrid zone between the golden-collared (*Manacus vitellinus*) and white-collared (*Manacus candei*) manakins, females prefer golden-collared males on mixed leks, which results in asymmetric introgression of golden plumage colouration across the hybrid zone (Parsons *et al.* 1993a; Stein & Uy 2006). In contrast,

evidence that divergence in sexual characters conferring an advantage in male-male competition can promote asymmetric gene flow between lineages is very limited (Hedrick 2013). This is despite that competition between males for resources is important for the evolution of character displacement and reproductive isolation (Grether *et al.* 2013), and hence features frequently in speciation theory (Price 2008). Behavioural experiments suggest that competitive exclusion of males of the sub-dominant lineage may contribute to the golden-collared male mating advantage at mixed leks in manakins (McDonald *et al.* 2001), cause differences in the frequency of hetero-specific pairings between pied and collared flycatchers (Vallin *et al.* 2012) and between hermit and Townsend's warblers (Pearson 2000). However, even in these relatively well-studied systems the link between intraspecific sexual selection and genetic and phenotypic introgression remains largely circumstantial.

We studied how behavioural interactions as well as post-copulatory and post-zygotic reproductive isolation influence gene flow between two phenotypically distinct lineages of the common wall lizard, *Podarcis muralis.* This species has formed a number of genetic lineages in Southern Europe reflecting isolation in ice age refugia (Salvi *et al.* 2013b). Our focus was on lizards native to Western Europe, which correspond morphologically to the *P. muralis brongniardii* subspecies, and on lizards native to northern Italy (Tuscany), which correspond morphologically to the *P. muralis nigriventris* subspecies (Böhme 1986). These lineages now form a natural contact zone in Liguria (northwestern Italy, see Results) and have also come into secondary contact more recently as a result of human introductions in both Germany and southern England (Schulte *et al.* 2012a; Michaelides *et al.* 2013).

We used an experimental approach to generate predictions regarding how natural and sexual selection should influence the direction of introgression, followed by genetic and phenotypic analyses in all three regions of secondary contact to test these predictions. First, we conducted an extensive phenotyping of lizards from the two main lineages in allopatric native populations

to establish the extent to which they exhibit divergence in sexually selected characters. Second, we used experimental populations in outdoor enclosures to test whether such differences translate into an asymmetry in male dominance and realized hybridization upon secondary contact. Third, we assessed the reproductive compatibility of the lineages and the survival and reproductive competence of F1 hybrids. Finally, we made use of these data to predict the direction of introgression, which we tested using mitochondrial DNA, microsatellites, and phenotypic data across three locations (one native and two introduced) of hybridization.

2.3 Materials and Methods

Common wall lizards are small, 45-75 mm snout-to-vent length, diurnal lizards that inhabit a range of natural and anthropogenic habitats. We studied native populations in France, western Germany, and northwestern Italy, which collectively belong to a mitochondrial lineage that we here refer to as the Western European lineage, and populations in northern Italy (Tuscany), which we refer to as the Italian lineage (Schulte *et al.* 2012a). The data in this paper also involve eleven introduced populations in England of known single native origin, and two introduced populations of mixed origin (i.e., presence of animals of both Western European and Italian origin or hybrids), one in England and one in Germany. Further details on the populations are found in Table S2.1.

(i) Character divergence in allopatry: We collected morphological and colouration data on 793 animals from 31 native populations of pure Western European and Italian origin. We captured all lizards by noosing, weighed them to the nearest 0.01 g and measured their snout-to-vent length, total length, head length and head width to the nearest mm. Using photographs, animals were scored for ventral (blackness) and dorsal (greenness) colouration. Ventral blackness was scored by quantifying the proportion of black to non-black pixels on each lizard's chest (Figure S2.1). Dorsal greenness was scored based on an intensity scale from 1 to 10 (1 being pure brown, 10 being pure green, Figure S2.2), which was confirmed to be highly correlated with scores from digital photographs analyzed in Photoshop CS4 and with values for green chroma extracted using spectrophotometry (see SI for full details). We also collected data on bite force and male testes mass, which are both commonly under sexual selection in lizards (Olsson & Madsen 1998; see SI for full details and sample sizes).

(ii) Patterns of dominance, courtship and paternity upon secondary contact: To generate predictions regarding the direction of hybridization we carried out two separate experiments

using outdoor enclosures (7×7 m), designed to simulate conditions during secondary contact. Each enclosure was fitted with suitable habitat (bricks, wooden pallets) and stocked with 16 animals four males and four females of each lineage (with the exception of 4 enclosures in 2010; see SI for details). In 2010, ten enclosures were stocked with a total of 160 animals of either Italian or Western European origin sourced from introduced populations in England. In 2013 we conducted a similar experiment using eight enclosures stocked with a total of 128 animals captured from native populations in western France or Tuscany. In both experiments we collected individuals from multiple populations (n = 10 in 2010 and n = 7 in 2013). To reduce population of origin effects, animals from the same source population were distributed among all enclosures as evenly as possible. Within this constraint the location of each individual was assigned randomly. The two experiments differed slightly in the distribution of habitat within enclosures, but followed the same protocol for data collection (see SI for full details).

Individuals were captured from the wild prior to females laying their first clutch and were transported to the laboratory. The experiments were conducted following oviposition of the first clutch (females typically lay at least two clutches per season). All individuals of each sex were released into a given enclosure at the same time. Males were released first to allow them to establish territories, followed by females (~7 days between the release of males and females). Females released more than three days after oviposition were kept cool (ca 10 °C) during this period to avoid progression through the next ovulation cycle. Behavioural interaction data were obtained throughout the experiment from rotating 45 minute observation periods per enclosure, conducted by three (in 2010) or two (in 2013) observers in an ethogram (Table S2.2, see SI for full details). This resulted in a total observation period of ~510 hours in 2010 and ~370 hrs in 2013.

Once females were ready to lay, all individuals were recaptured and returned to cages in the laboratory. Cages were inspected in the morning and late afternoon for signs of egg laying. Eggs

were incubated at a constant 24°C (2010) or 28°C (2013) in standard refrigerated incubators fitted with water baths to maintain humidity. At hatching, offspring were euthanized (using concussion followed by permanent destruction of the brain) and their tissues used for genetic analysis. DNA was isolated from tail-tip tissue using standard protocols (see SI for full details). Paternity was assigned using microsatellites in CERVUS v 3.0 (Marshall *et al.* 1998) based on the trio (mother, father, offspring) LOD score and a strict confidence level of 95%.

To confirm whether these patterns were the result of pre- as opposed to post-copulatory mechanisms we carried out 16 sperm competition trials in the laboratory in which Western European (n = 6) and Italian (n = 10) females were mated to males of both their own and the other lineage. All trials were carried out in the same type of terrarium used for housing the animals (see above) in the first 5 days following oviposition, which corresponds to the female receptive period under laboratory conditions. Females were introduced to the terrarium and allowed to acclimatize for twenty minutes after which time one of the males was introduced to the female. Once mated (all within an hour) that male was removed and the second male was immediately introduced (all also mated within an hour). The order of males with respect to lineage was reversed each trial. Offspring were genotyped along with their mother and the two potential fathers as described above.

(iii) Fertility and viability of F1 hybrids. To test for decreased hybrid fitness we carried out 62 crosses between males and females of the two lineages. We introduced a male of either the same lineage or the other lineage into a female cage three days after she had laid her first clutch and left them together for 5 days. Eggs were collected following oviposition, scored for infertility based on presence and calcification of the egg shell (Olsson & Shine 1997a) and incubated at 24°C. Embryonic mortality was scored and assessed using dissection of eggs that did not show any evidence of heart beat (using a digital egg monitor: Buddy, Avitronics, England). Ninety-six offspring from these crosses were raised to maturity under laboratory conditions. After

reaching mature size (~ five months after hatching) they were hibernated for ten weeks at 4°C. For logistical reasons we were unable to conduct further crosses to establish a F2 generation and therefore assessed reproductive capacity under captive conditions for a subset of animals. We recorded whether or not females produced eggs within two months of emergence of hibernation and the resulting clutch size. We also recorded the testes mass of 23 males. These characters should reflect fertility of F1 hybrids, but it should be noted that it does not establish sperm characteristics in males and that incompatibilities may not be evident until the F2 generation; our data may therefore underestimate genetic incompatibilities in hybrids.

(iv) Statistical analyses. All data were analysed using R v.3.03 (R Development Core Team 2010). We used linear (mixed) models to analyse differences between lineages and sexes in phenotypic characters in both wild-caught animals and experimental crosses and to establish patterns of behaviour and parentage in the experimental enclosures. Detailed description of all models can be found in the SI.

(v) Genetic and phenotypic patterns in regions of secondary contact. We examined phenotypic and genetic patterns of introgression within three separate regions of secondary contact between the Italian and Western European lineage. In the native hybrid zone, we sampled 17 populations from central Tuscany (where animals are known to fall within the Tuscan haplotype lineage (*sensu* Schulte *et al.* 2012a) and exhibit *P. m. nigriventris* phenotypes) to western Liguria (where animals are known to belong to the western European haplotype lineage and exhibit typical *P. m. brongniardi* phenotype) (Böhme 1986, Figure 2.1).

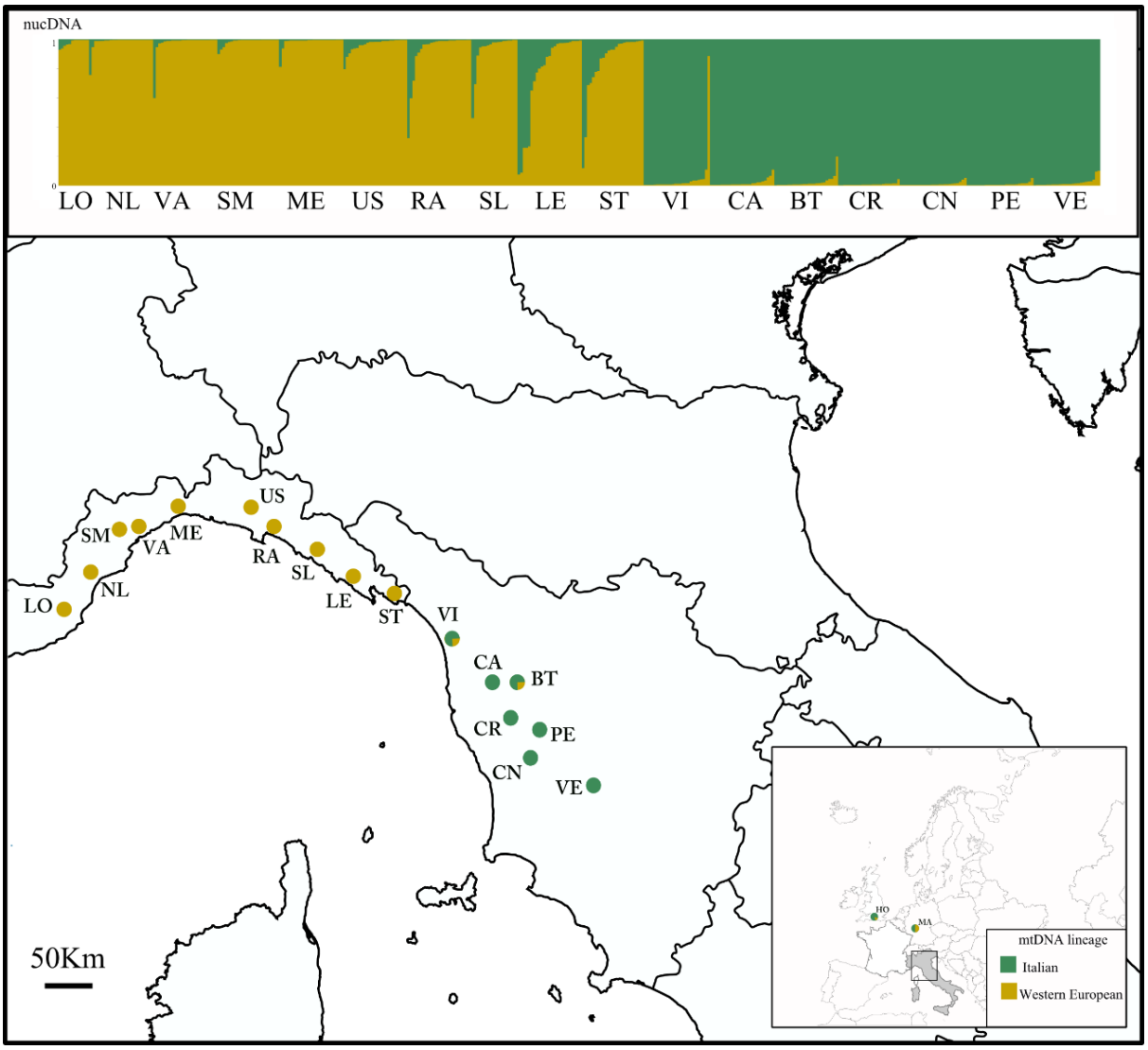

Figure 2.1: Map of the native hybrid zone in northern Italy. We used 17 populations that created a cline from mid Tuscany up the Ligurian coast. The colour of the dots indicates the association with a particular mitochondrial lineage. Two populations in the middle of the cline contained a mix of haplotypes (VI and BT). At the top of the figure a STRUCTURE output indicates the extent of admixture (using microsatellite nuclear DNA) within these populations as a function of distance from the far western end of the cline (running left to right). Insert shows the location of the geographic region and the two hybrid regions in England (Holmsley) and Germany (Mannheim).

Second, we sampled 27 animals from a location in England (Holmsley) that is known to have both Italian and Western European origins (Michaelides *et al.* 2013). Third, we sampled 203 animals from a population in south-western Germany (Mannheim) where animals from the Italian lineage have been introduced in a region where the western European lineage is native

(Schulte *et al.* 2012a). In each of these populations, we recorded traits as described above and removed ca 5 mm of the tail or took buccal swabs for DNA analyses.

In the native hybrid zone, we tested predictions regarding the direction of gene flow using a geographic cline approach (Szymura & Barton 1986a; Gay *et al.* 2008b). Because microsatellite loci were highly variable and showed few private alleles, typically at low frequencies, we estimated the nuclear genetic cline from a Bayesian hybrid index (HI) based on allele frequencies at all loci using the program STRUCTURE v 2.3.4 (Pritchard *et al.* 2000). Because phylogeographic studies have established two lineages in this geographic region we conducted all analyses assuming two genetic clusters (i.e., K=2). The simulations, using the admixture model, run with a burn-in of 10^5 iterations and a further run length of 10^6 iterations. Runs were replicated five times and combined using CLUMPP (Jakobsson & Rosenberg 2007). We used the probability that an individual was assigned to the Italian cluster (Q) as our hybrid index. The hybrid index was subsequently used to assign individuals as either pure Western European ($Q \leq 0.1$), pure Italian ($Q \geq 0.9$), or hybrid ($0.1 < Q < 0.9$) (e.g., Baldassarre *et al.* 2014). We also fitted the corresponding cline for haplotypes based on the cytochrome *b* mitochondrial gene. Phenotypic clines using population averages were fitted for three traits; dorsal greenness, ventral blackness, and relative head length. These are all quantitative characters with large and well-established differences between lineages (greater in the Italian lineages, e.g., Böhme 1986, Figure S2.3; see Results). Relative head length was calculated as the residual score from a regression of head length on snout-to-vent length. Clines were treated separately for males and females.

Genetic and phenotypic clines were fitted using the Metropolis-Hastings Markow chain Monte Carlo algorithm implemented in the package hzar in R version 3.0.3 (Derryberry *et al.* 2014). For the genetic analyses we ran two sets of five models. Each model estimated cline centre (cumulative distance from sampling location Colle di Val D'Elsa in Tuscany, *c*) and width

(1/maximum slope, *w*), but could also fit different combinations of the exponential decay curve parameters δ and τ (none, right tail only, left tail only, mirrored tails, or both tails separately). One set of models fixed the cline ends at the empirically observed values, whereas the other set also estimated these values from the data. We also ran the corresponding models for each of the phenotypic traits, but because of small sample size (<10) for the Westernmost population, the models with fixed cline ends used the value of the closest population (i.e., Noli, NL). For each of the clines we compared models based on the AIC corrected for small sample size (AICc) and selected the model with the lowest AICc as the best-fitting model. The coincidence of cline centres for mtDNA vs. ncDNA, and for ncDNA vs. phenotypic clines was assessed using the maximum-likelihood derived confidence intervals, where we considered non-overlapping confidence intervals as statistically supported differences in cline location. We verified the conclusions by re-fitting models that constrained the cline centre to correspond to that of the cline to which it was compared.

For the two non-native populations we tested for the presence of hybrids and the direction of hybridization. First we conducted a Principle Coordinate Analysis (PCoA) to visualize pairwise individual multilocus genetic distance calculated in GenAlEx (Peakall & Smouse 2012). For the non-native population in England, we included four non-native populations of pure origin (three of Italian and one of Western European origin) that served as source populations (Michaelides *et al.* acc. pending revisions). For the non-native population in Germany (Mannheim) we did not include reference populations as the exact origins are unknown. Instead, we pooled individuals into two groups based on the lineage assignment from the cytochrome *b* gene (Western European and Italian). Secondly, we conducted Bayesian assignment tests to identify individuals of mixed origin. We used the admixture model as implemented in STRUCTURE to assign individuals as either pure Western European ($Q \leq 0.1$), pure Italian ($Q \geq 0.9$), or hybrid ($0.1 < Q < 0.9$). We also did the corresponding analysis in the program NewHybrids (Anderson & Thompson 2002), which computes posterior probabilities of individual assignment into

different categories of hybrids based on their multilocus genotypes (F1 or F2). We used uniform priors with 10^5 sweeps before and 10^6 sweeps after burn in. The direction of hybridization was assessed by comparing the outcome of these assignment tests to the mitochondrial haplotype.

2.4 Results

(i) Character divergence in allopatry: Lizards from the Italian lineage displayed the characteristic green dorsal and black ventral colouration typically ascribed to *P. m. nigriventris* and were larger with larger heads, stronger bite force, and greater testes mass (Table S2.4; Figure 2.2). Sexual dimorphism was generally greater in the Italian lineage (Table S2.4, Figure 2.2).

(ii) Patterns of paternity upon secondary contact: We found highly consistent results across both experiments. Italian males were strongly dominant over Western European males, winning more agonistic interactions (permutation test using QAP – 2010, lineage: $P = 0.019$, snout-to-vent length: $P = 0.012$, 2013, lineage: $P < 0.001$, snout-to-vent length: $P = 0.05$; Figure 2.3a). Across both experiments, dorsal greenness, ventral blackness and head length, phenotypic characters that are exaggerated in Italian males, were all strong phenotypic predictors of dominance (Table S2.5).

Italian males courted significantly more females (Table S2.6; Figure 2.3b), and had higher reproductive success overall and with females of the opposite lineage, than Western European males (Table S2.6; Figure 2.3c). Accordingly, Western European females produced a significantly higher proportion of hybrid offspring compared to Italian females (Table S2.6; Figure 2.3d). Rerunning models including dominance as a predictor suggested that differences in reproductive success between Italian and Western European males were well explained by dominance and hence consistent with male-male competition (Table S2.7).

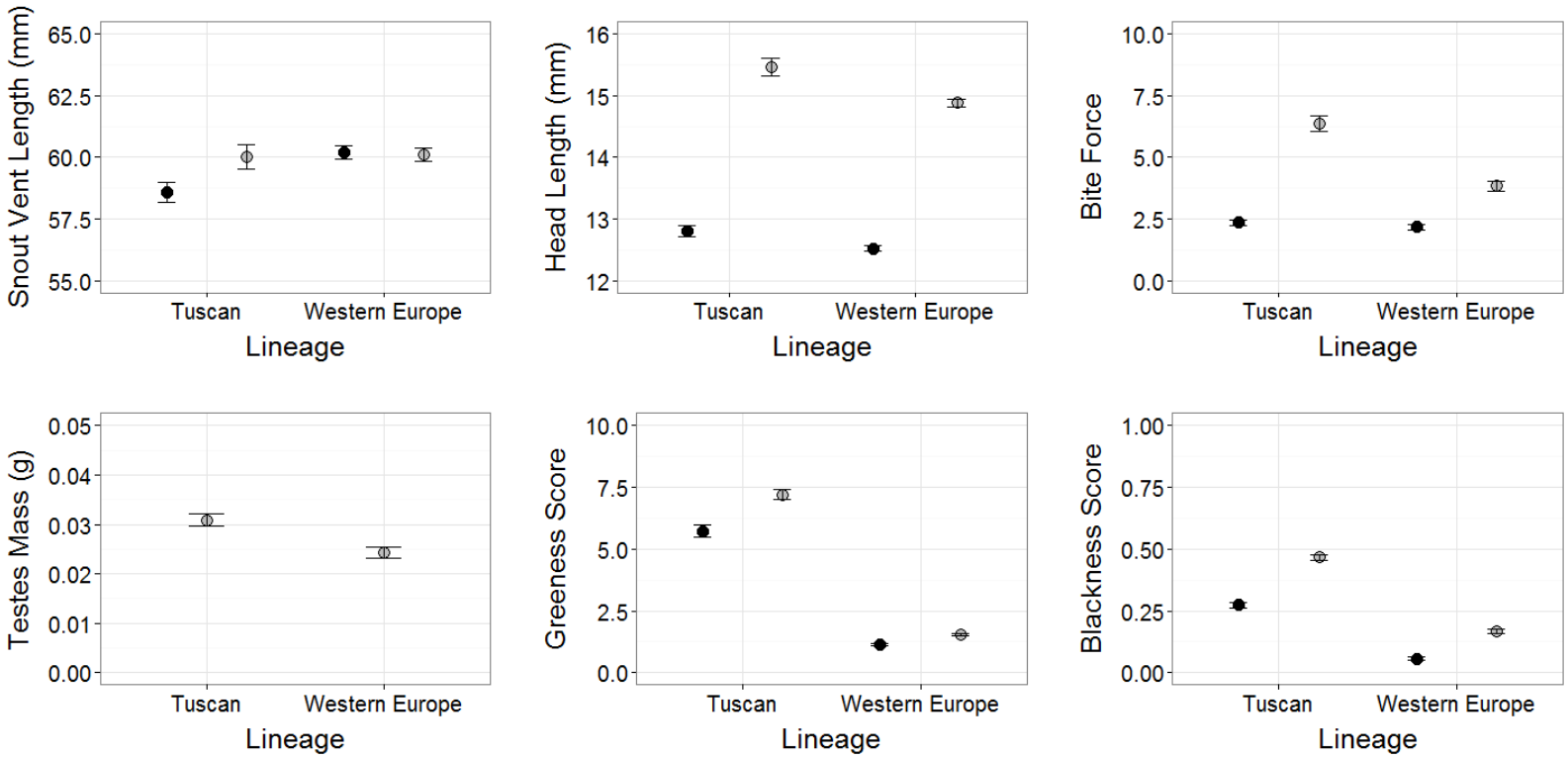

Figure 2.2: Means ± SE for Western European and Italian lizards in morphological and colour phenotypes. Black dots indicate females and grey dots indicate males.

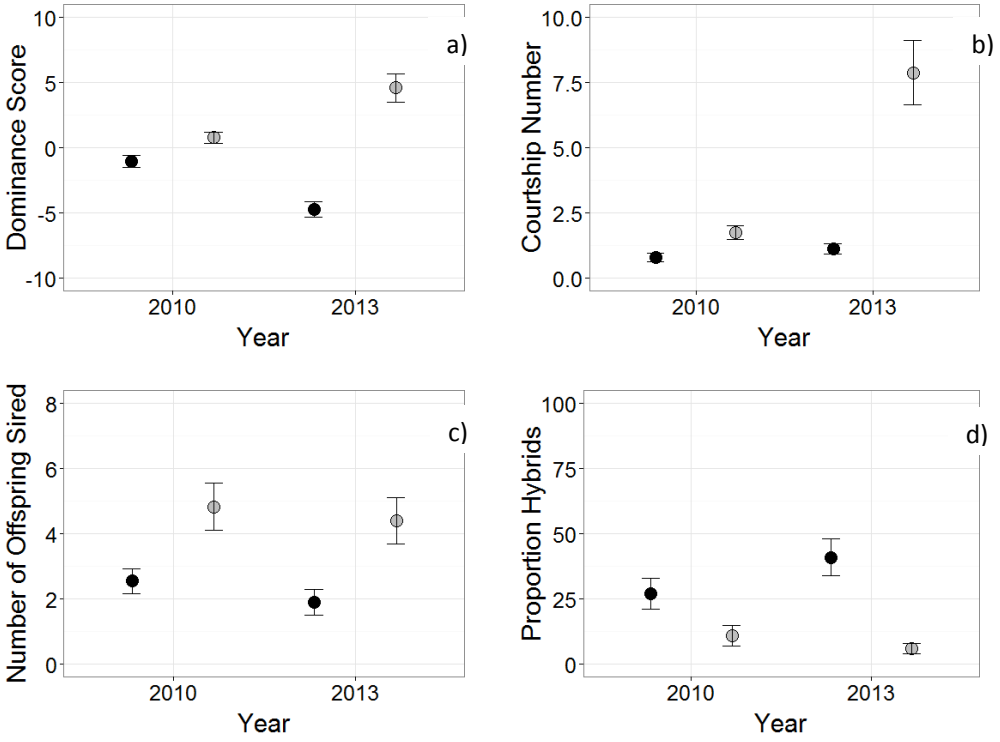

Figure 2.3: Means ± SE for Western European and Italian lizards in male dominance, number of courtships males initiated, the number of offspring males sired and the proportion of a females clutch that were hybrids. All outputs are the result of our experimental secondary contact zone experiments carried out with non-native (2010) and native (2013) lizards. Black dots indicate Western European lizards and grey dots indicate Italian lizards. Note that the higher reproductive success of Italian males (panel c) is the result both of a greater number of clutches with paternity assignment for Italian females and higher reproductive success with females of the opposite lineage (panel d).

Males were more likely to sire offspring with females from their own lineage under sperm competition (intercept: $\chi^2 = 8.45$, df = 1, $P < 0.01$), but there was no statistical support for a bias in reproductive success with females from the other lineage between Western European and Italian males (proportion of hybrids in Italian clutches = 0.21 ± 012, proportion of hybrids in Western European clutches = 0.42 ± 0.20, lineage: $\chi^2 = 0.22$, df = 1, P = 0.64).

(iii) Fertility and viability of F1 hybrids. Embryonic mortality was not higher for between-lineage crosses (17%) compared to within-lineage crosses (16%) (male lineage: $\chi^2 = 3.19$, P = 007, female lineage: $\chi^2 = 3.19$, P = 0.43, male lineage×female lineage: $\chi^2 = 0.42$, P = 0.51). Animals of pure Western European origin had slower growth rates and were smaller following hibernation than animals of Italian and hybrid origin (Western European offspring = 47.3 ± 1.19 mm, Italian offspring = 52.5 ± 0.78 mm, Hybrid offspring = 51.6 ± 0.64 mm, cross: $\chi2 = 245.3$, $P < 0.001$, sex: $\chi2 = 16.8$, P = 0.32). We found no significant difference between the crosses in testes length for a given body size (Western European males = 0.017 ± 0.01g, Italian males = 0.035 ± 0.01g, Hybrid males = 0.031 ± 0.01, cross: $\chi^2 = 1.81$, P = 0.24, snout-to-vent length: $\chi^2 = 0.07$, P = 0.72). Captive-reared females of French origin did not reproduce, but female hybrids were as likely to reproduce as pure-bred Italian females (36% of Italian females reproduced vs 45% of hybrid females: $\chi^2 = 0.03$, P = 0.85), and there was no significant difference in clutch size (Italian females = 3.20 ± 0.58, Hybrid females = 3.55± 0.17: $\chi^2 = 0.51$, P = 0.48).

(iv) Genetic and phenotypic patterns in regions of secondary contact: The results above predict that introgression should be male-driven and asymmetric from the Italian lineage into the Western European lineage. As predicted, the location of the geographic cline for microsatellites was shifted westwards compared to the cline for mtDNA (Table 2.1; Figure 2.4). A microsatellite cline with its centre constrained to that of the mtDNA cline provided a significantly worse fit to the data (ΔAICc =

43.1). The locations of both genetic clines were significantly different from the cline centres for all three phenotypic traits (dorsal greeness Figure 2.4, ventral blackness and relative head length Figure S2.4), which were shifted even further to the west (Table 2.1). Notably, we failed to identify significant levels of genetic admixture for several of the western-most populations with Western European haplotypes (e.g., US, RA, SL: Figure 2.1) that were phenotypically very similar to populations identified as being of pure Italian origin (Figure 2.4). The best-fitting models for the phenotypic clines differed between traits and, for black ventral colouration, between the sexes (Table S2.8). There was a significant correlation between genetic differentiation and geographic distance ($r^2 = 0.70$, $P < 0.001$, Figure S2.5).

STRUCTURE assigned all individuals in the putative hybrid population in southern England as being of pure Italian origin despite four individuals having mtDNA haplotypes from Western Europe (Figure S2.6). The results were corroborated by the output from NewHybrids in which all individuals were assigned as being pure Italian (Table S2.9) and the Principal Coordinate Analysis (PCoA) in which hybrid individuals were found within the cluster of pure Italian individuals (Figure S2.7). In Mannheim, STRUCTURE identified 23 out of 203 individuals as hybrids ($0.1 < Q < 0.9$; Figure S2.8) and NewHybrids tended to classify these as being F2 hybrids (e.g., F1 x F1 hybrids; Table S2.11). Eight hybrid individuals in Mannheim harbored Italian mtDNA haplotypes and the rest (15) had mtDNA haplotypes from Western Europe (Table S2.10). The PCoA placed these within and/or between the clusters of pure individuals (Figure S2.9).

Table 2.1: Parameter estimates for best fitting cline models for genetic and phenotypic clines using *HZAR* (Derryberry *et al.* 2014). Parameter *c* indicates the estimated cline centre (distance from sampling location VE in Tuscany) and *w* indicates the cline width (1/maximum slope). The parameters *pmin* and *pmax* indicate the allele frequencies at the ends of the cline for genetic markers and the corresponding values for phenotypic markers (transformed values to the second decimal point), and δ and τ are exponential decay curve parameters for the left and right tails. Two log-likelihood unit support limits are presented in parentheses. Note that the very high introgression of phenotypic characters makes the parameter estimates for the fit in the western part of the cline unreliable (see Figure 2.4 and Figure S2.4)

Character	Sex	Best Model	c (km)	w (km)	pmin	pmax	δL	τL	δR	τR
mtDNA		Model I	61.2 (56.0-68.8)	29.9 (18.3-50.3)	0	1	None	None	None	None
Hybrid Index		Model VII	100.5 (88.7-118.7)	15.2 (1.2-50.2)	0	1	None	None	2.37 (0.0-18.2)	0.131 (0.011-0.705)
Greenness	M	Model II	273.0 (254.3-278.0)	76.1 (56.2-93.6)	1.44	2.71	None	None	None	None
Greenness	F	Model II	228 (225.7-230.0)	5.1 (3.7-5.9)	0.07	1.82	None	None	None	None
Blackness	M	Model II	156.2 (124.3-279.8)	105.7 (20.1-309.3)	0.18	0.45	None	None	None	None
Blackness	F	Model VIII	226.6 (219.7-231.5)	22.1 (14.7-56.7)	0.06	0.23	None	None	292.4 (1.2-307.4)	0.743 (0.006-0.972)
Head length	M	Model I	213.2 (207.2-222.8)	14.8 (0.1-30.2)	-0.38	0.14	None	None	None	None
Head length	F	Model I	227.5 (215.2-238.9)	62.6 (25.4-125.7)	-0.72	0.08	None	None	None	None

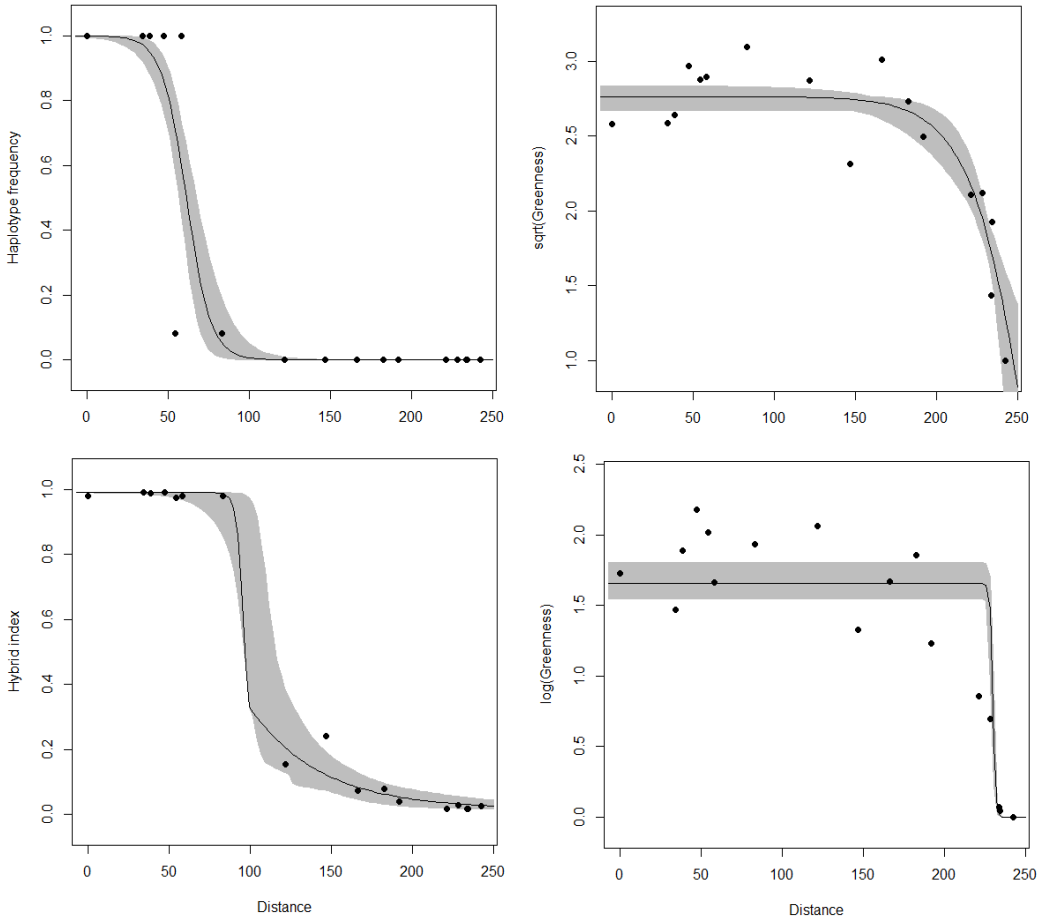

Figure 2.4: The maximum likelihood cline and the 95% credible cline region for best-fitting models (Table 2.1) for mtDNA haplotype (top left), hybrid index (bottom left) and the lineage-characteristic dorsal colouration for males (top right) and females (bottom right). Greenness of dorsal colouration was scored on a scale of one to ten and was transformed to improve fit to model assumptions (square rote and logarithmic transformation for males and females, respectively). Transect distance is the cumulative distance from the south-easternmost population Colle di Val D'Elsa in Tuscany with increasing distance westwards.

2.5 Discussion

The evolutionary consequences of secondary contact should depend on the genetic and phenotypic divergence between lineages. Our results show that divergence in male competitive ability in allopatry causes asymmetric hybridization and gene flow upon secondary contact in wall lizards. As a consequence, sexually selected introgression shapes phenotypic and genetic variation in both native and non-native populations.

Wall lizards in north-central Italy show exaggeration of characters that are under sexual selection in this (Sacchi *et al.* 2009a) and other lizard species (Olsson & Madsen 1998a). Our experiments show this is associated with an advantage in male-male competition for females, leading to an overall greater courtship, mating success and increased rates of hybridization with females of the other lineage compared to males from the Western European lineage. These patterns are unlikely to be mediated by female choice as we have shown elsewhere that females do not discriminate between males of different lineages whereas males prefer females from their own lineage (Heathcote 2013). Post-copulatory mechanisms also appear unlikely to explain patterns of paternity as there was no evidence for a competitive advantage for Italian males in sperm competition trials. Our results therefore suggest that male-male competition and male mate choice should drive patterns of genetic exchange in zones of secondary contact. This is in contrast with the majority of previously studied vertebrates, where female choice is believed to be both the primary barrier to hybridization as well as the major cause of asymmetric introgression of male characters (McMillan *et al.* 1997; Wirtz 1999a; Stein & Uy 2006; Pfennig 2007; van der Sluijs *et al.* 2008).

Even if small differences in viability or fertility remained undetected in our experimental crosses, the differences in male competitive ability should create asymmetric introgression; i.e., male-driven gene flow from the Italian lineage into the Western European lineage. Data from

the native hybrid zone provide strong support for this prediction. Nuclear microsatellite markers revealed a westward shift in the position of the hybrid cline compared to mitochondrial markers. As a result virtually all hybrids exhibited Western European haplotypes. Furthermore, the phenotypic clines were shifted even further westward such that several populations that were genetically (i.e., based on both ncDNA and mtDNA) assigned to the Western European lineage were phenotypically indistinguishable from pure populations of the Italian lineage. Even if head size and dorsal and ventral colouration are not the direct targets of sexual selection, our enclosure experiments show that these characters are strong predictors of male dominance, a robust predictor of reproductive success. Thus, the stronger introgression of phenotypic characters compared to microsatellite markers imply that these characters not only bias the direction of hybridization but are also selectively favoured within the hybrid zone. However, analysis of selection at the leading front of the hybrid zone would be necessary to establish ongoing selection on male secondary sexual characters.

The results from the native hybrid zone were supported by genetic data from two locations where at least one of the lineages has been introduced. These patterns were weaker than those observed in the native zone, potentially because of strong founder effects that are likely to have occurred during establishment. Nevertheless, in both of the non-native populations the mitochondrial-nuclear discordance was consistent with hybridization being primarily between males of the Italian lineage and females of the Western European lineage. Thus, the results from all three regions of secondary contact point towards asymmetric introgression and displacement of male characters of the less dominant lineage by intra-sexual selection (Schulte *et al.* 2012b), providing evidence that introgression can be a source of secondary sexual characters.

Is male-male competition a general mechanism of directional introgression? Differences in male competitive ability are commonly invoked to explain displacement of one species by another in

sympatry (Grether *et al.* 2013). This could promote asymmetric hybridization by making males of one species rare relative to females (Hubbs 1955). This mechanism is supported by studies of interspecific competition over nest sites in flycatchers (Vallin *et al.* 2012), but introgression in this species is very limited due to low hybrid fitness (Veen *et al.* 2001). Species-specific male aggression is also consistent with the direction of introgression of plumage colour in manakins (McDonald *et al.* 2001), the movement of hybrid zones between hermit and Townsend's warblers (Pearson & Rohwer 2000) and between two species' of house mice (Teeter *et al.* 2007). Nevertheless, the best evidence that sexual selection drives introgression still comes from studies of female choice (Parsons *et al.* 1993; Stein & Uy 2006; Baldassarre & Webster 2013; Baldassarre *et al.* 2014). This could partly be because of taxonomic bias. In lizards, male-male competition appears to be a stronger driver of variation in male reproductive success than female choice (Olsson & Madsen 1995; Font *et al.* 2012a). We therefore suggest that male-male competition often will be more important for the strength and direction of gene flow in lizard hybrid zones compared to, for example, bird hybrid zones.

The clines we observe in wall lizards are wider relative to the species dispersal ability than in other studies of sexually selected introgression (e.g., *Manacus sp.*; Uy & Stein 2007). In manakins, plumage introgression has been suggested to be limited by either habitat, which influences the conspicuousness of colour and geographically limits the benefit of golden plumage (Uy & Stein 2007), or by geographic barriers to dispersal (McDonald *et al.* 2001). In contrast, the habitat across the hybrid zone in the wall lizards typically consists of rocks and man-made structures (e.g., dry-stone walls), and geographic difference in the properties of this habitat are unlikely. Thus, there may be no limit to introgression along the coast in northwestern Italy and the geographic cline may be best viewed as a snap-shot of an ongoing process of adaptive introgression that will eventually replace the phenotypes of the Western European lineage in this part of the species' distribution. In the introduced populations, we

expect the formation of a hybrid swarm biased towards Italian characteristics, a process that evidently has already taken place in Holmsley.

Despite the close fit between our experimental data and the genetic and phenotypic clines, discordances between markers could also arise for a number of other reasons. We can refute most, if not all, of these for the native hybrid zone. First, the geographic scale of the discrepancy, compared to species dispersal distances, makes sex differences in dispersal highly unlikely as the cause of asymmetric introgression (Petit & Excoffier 2009). Lizard densities are also uniformly high across the zone. Second, we found no evidence that hybrid females are sterile, which rules out loss of fitness in female hybrids explaining differences in the mitochondrial and nuclear genetic clines (as expected from Haldane's rule; Haldane 1922). Third, environmental differences cannot explain the geographic pattern of phenotypic variation since the lineage differences persist in non-native populations and in captivity. Finally, the quantitative nature of the phenotypic characters means that it is unlikely that we are observing stochastic variation in introgression of loci across the genome, as could be the case for characters controlled by a single locus (e.g., colour polymorphisms; Sinervo *et al.* 2001; Rosenblum *et al.* 2004; Mundy 2005).

In summary, we provide strong evidence that divergence in sexually selected traits in allopatry drives asymmetric hybridization in wall lizards. This creates pronounced discordance between the phylogeography inferred from genetic markers and geographic patterns of phenotypic variation across multiple zones of secondary contact. These results suggest that, where post-reproductive isolation evolves slowly and female choice on male quantitative traits is absent or weak (as in lizards; Olsson & Madsen 1995), male-male competition may be an important cause of asymmetric introgression. This can lead to rapid introgression of potentially advantageous alleles and traits between species and ultimately promote novel genetic and phenotypic diversity in recipient populations.

2.6 Acknowledgments

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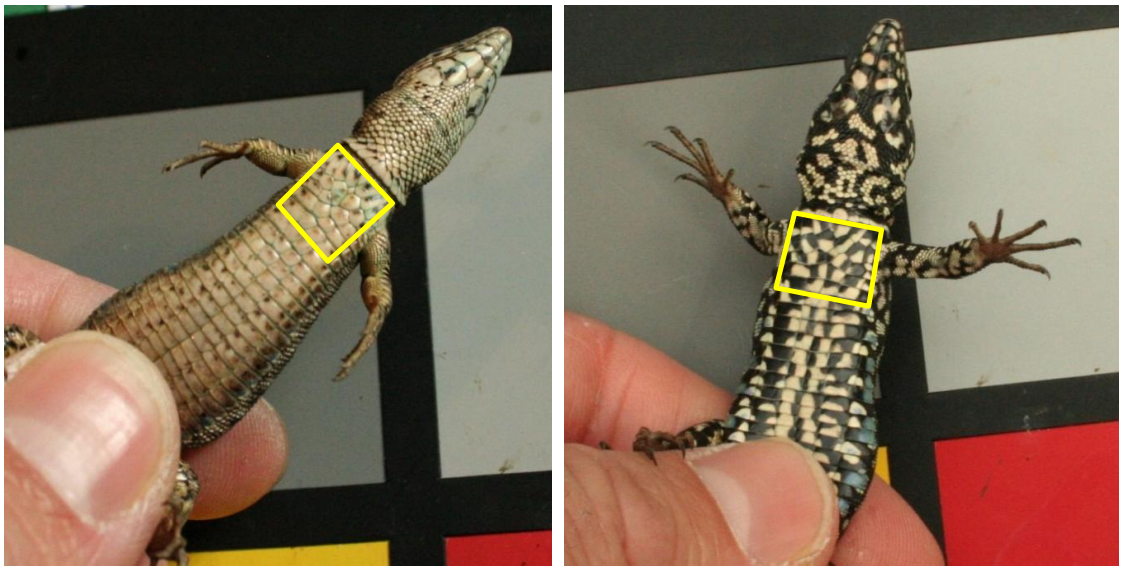
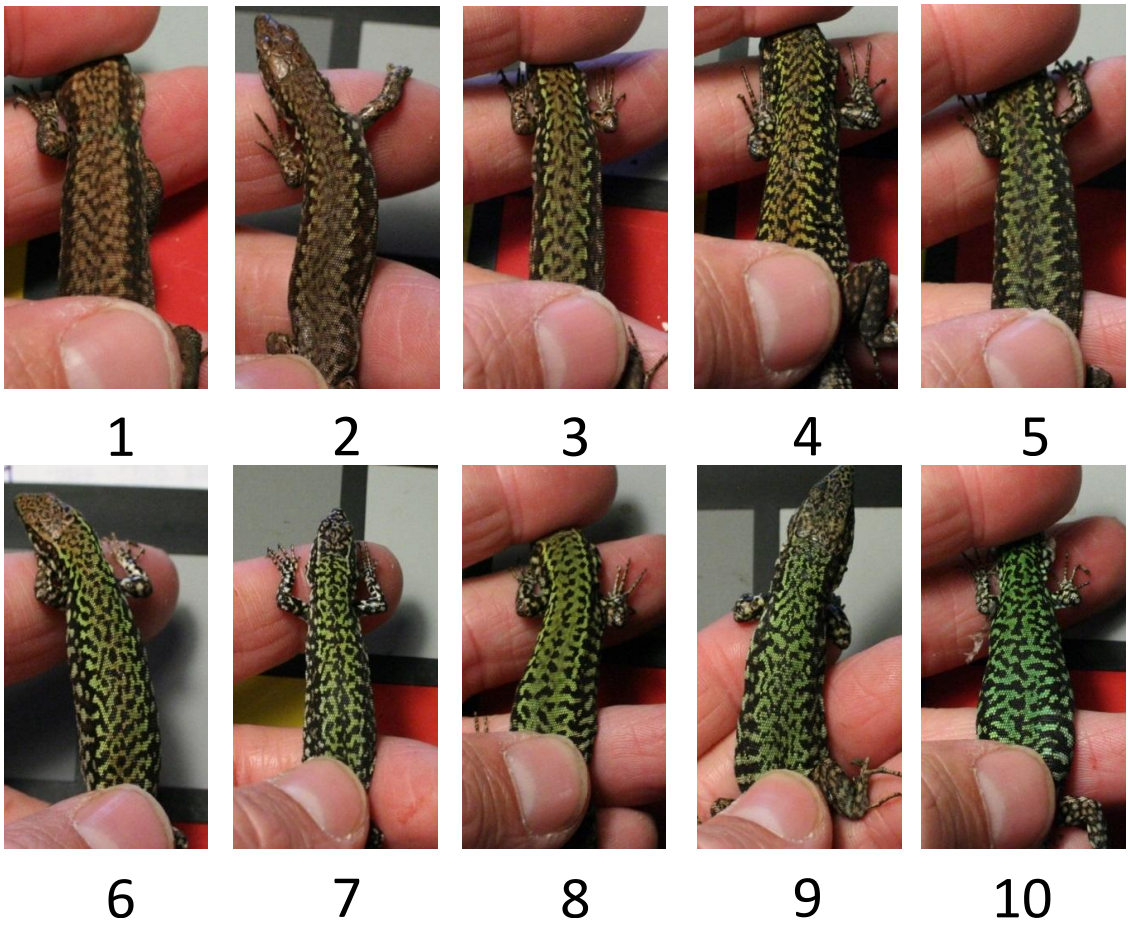
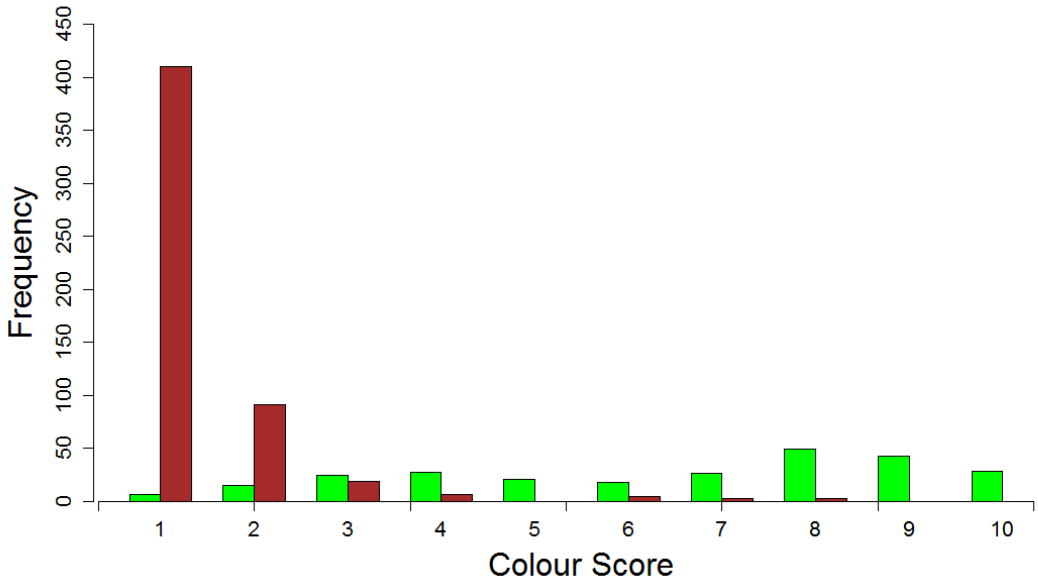
2.8 Supplementary Figures



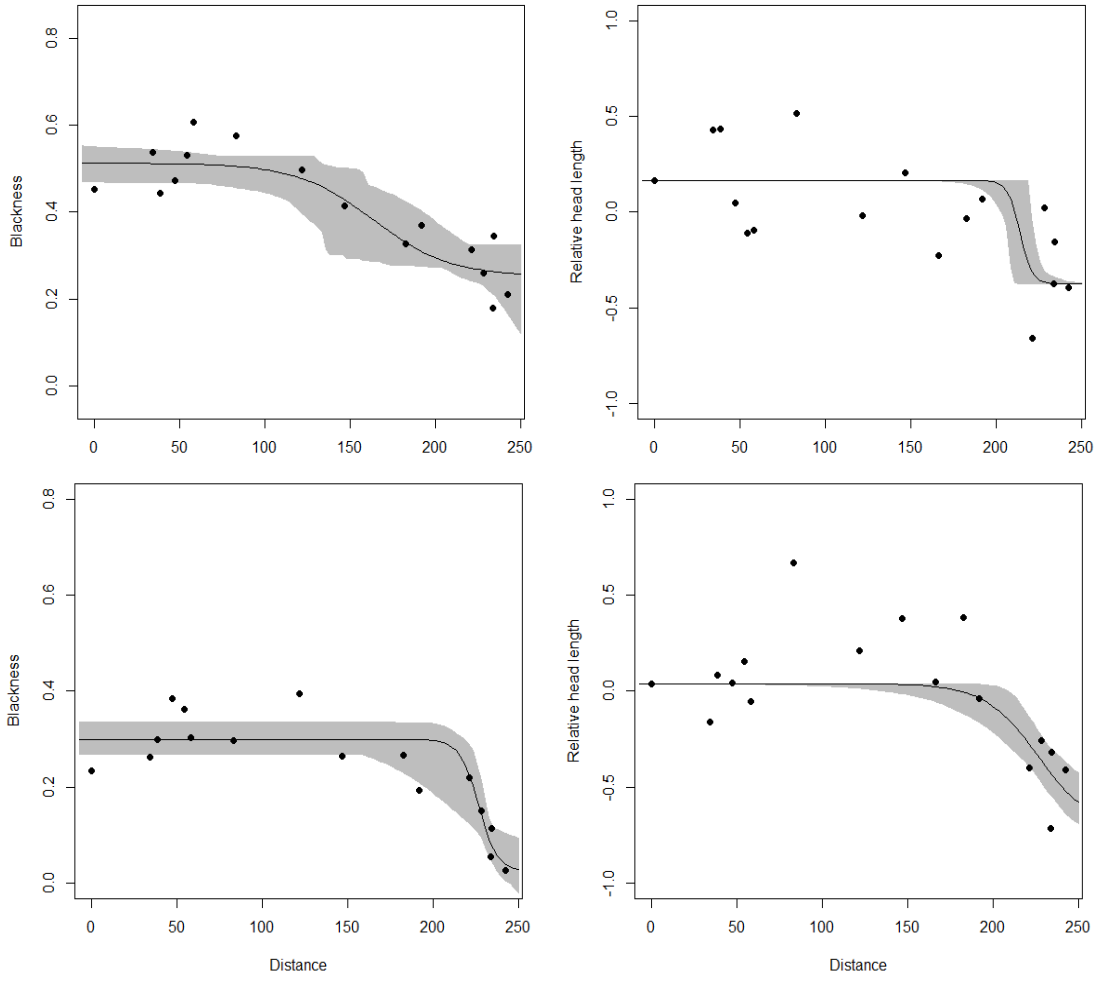
Supplementary Figure S2.1: Photos indicating the area of the ventral surfaced used to quantify blackness.



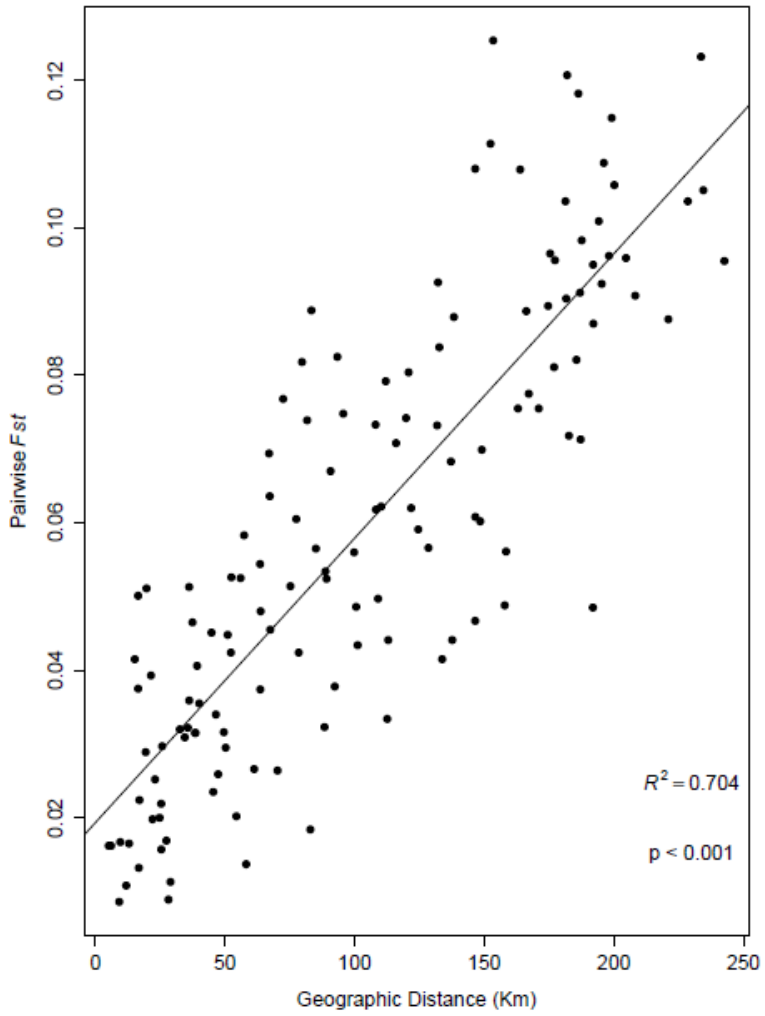
Supplementary Figure S2.2: Photos representative of the dorsal greeness scores assigned to each of the lizards. Dorsal greenness was scored based on intensity of greenness on a scale of 1 to 10. These scores were confirmed to be highly correlated with objective colouration scores (see above).



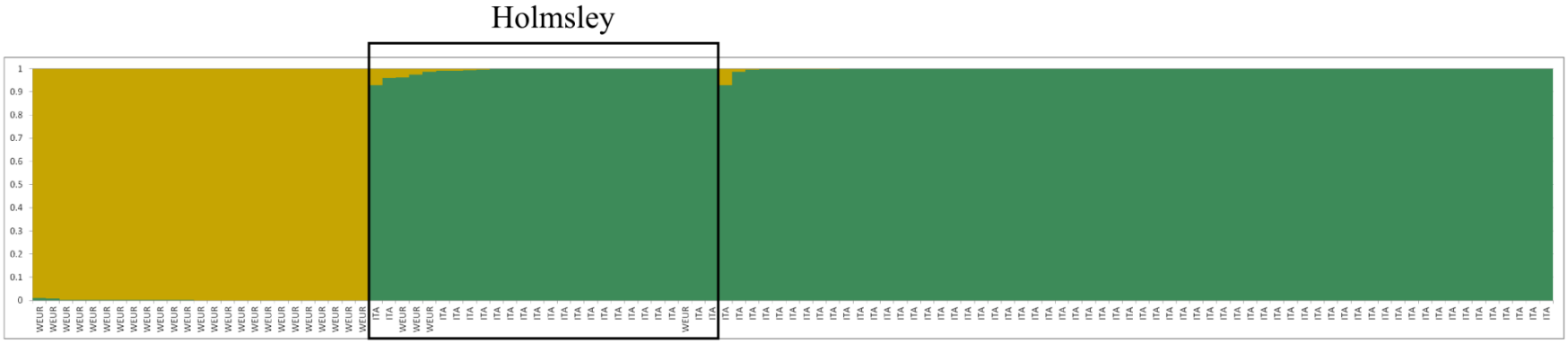
Supplementary Figure S2.3: Frequency distribution for dorsal greeness scores in Western European (brown) and Italian (green) populations of pure origin.



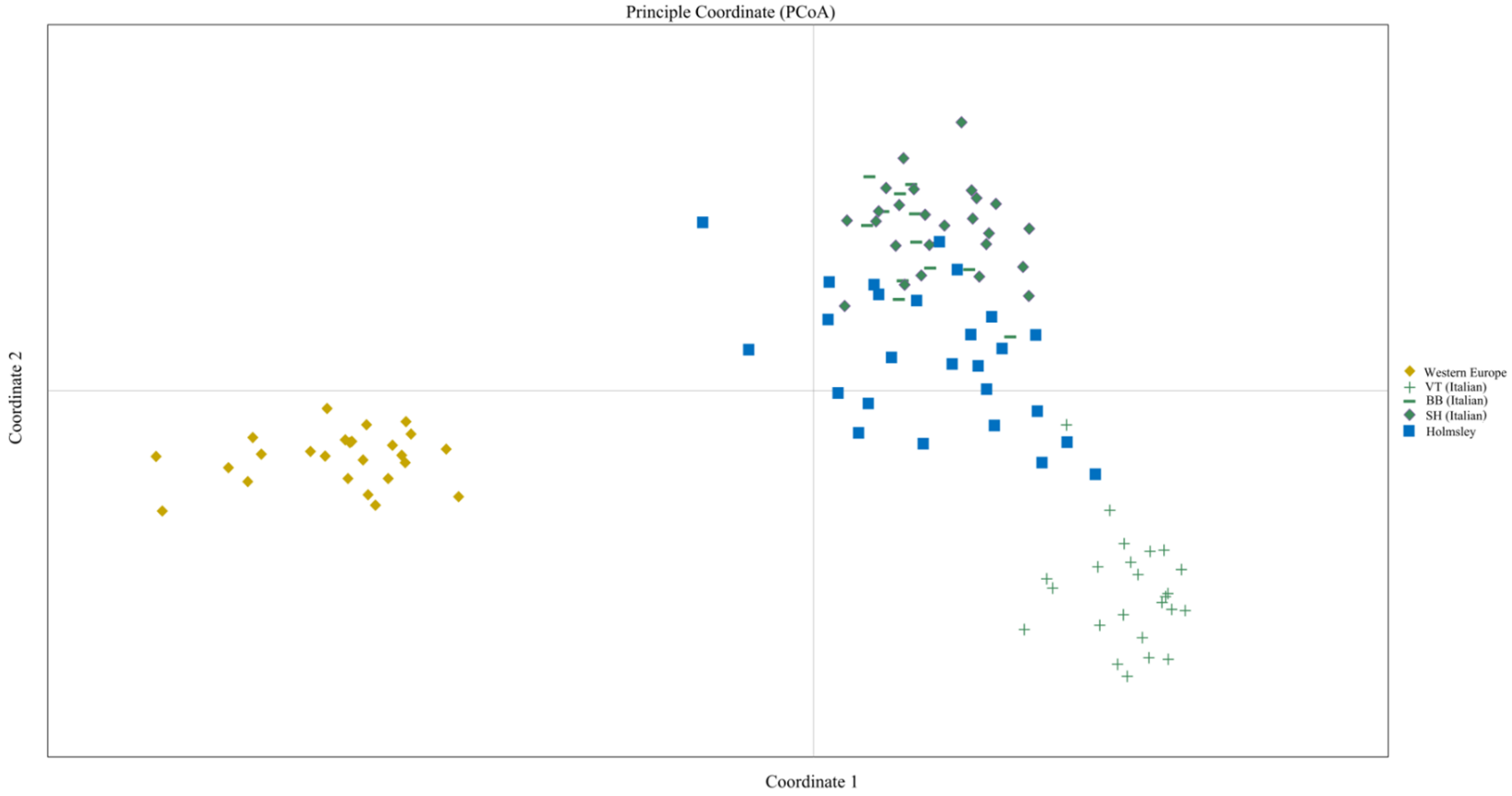
Supplementary Figure S2.4: The maximum likelihood cline and the 95% credible cline region for best-fitting models for male (top) and female (bottom) black ventral colouration (left panels) and relative head length (right panels) (see Table S2.5 for AICc for different models and Table 2.4 for parameter estimates for best-fitting models). Higher values for blackness and relative head length correspond to a greater proportion of the chest area that is black and a larger relative head width. Transect distance is cumulative distance from the south-easternmost population, Colle di Val D'Elsa in Tuscany, with increasing distance westwards



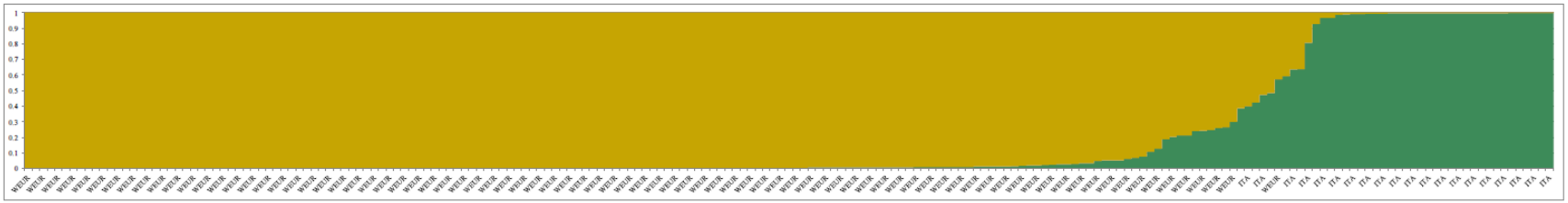
Supplementary Figure S2.5: Relationship between geographic distance and genetic differentiation (F*st*) for all populations used in the cline analysis.



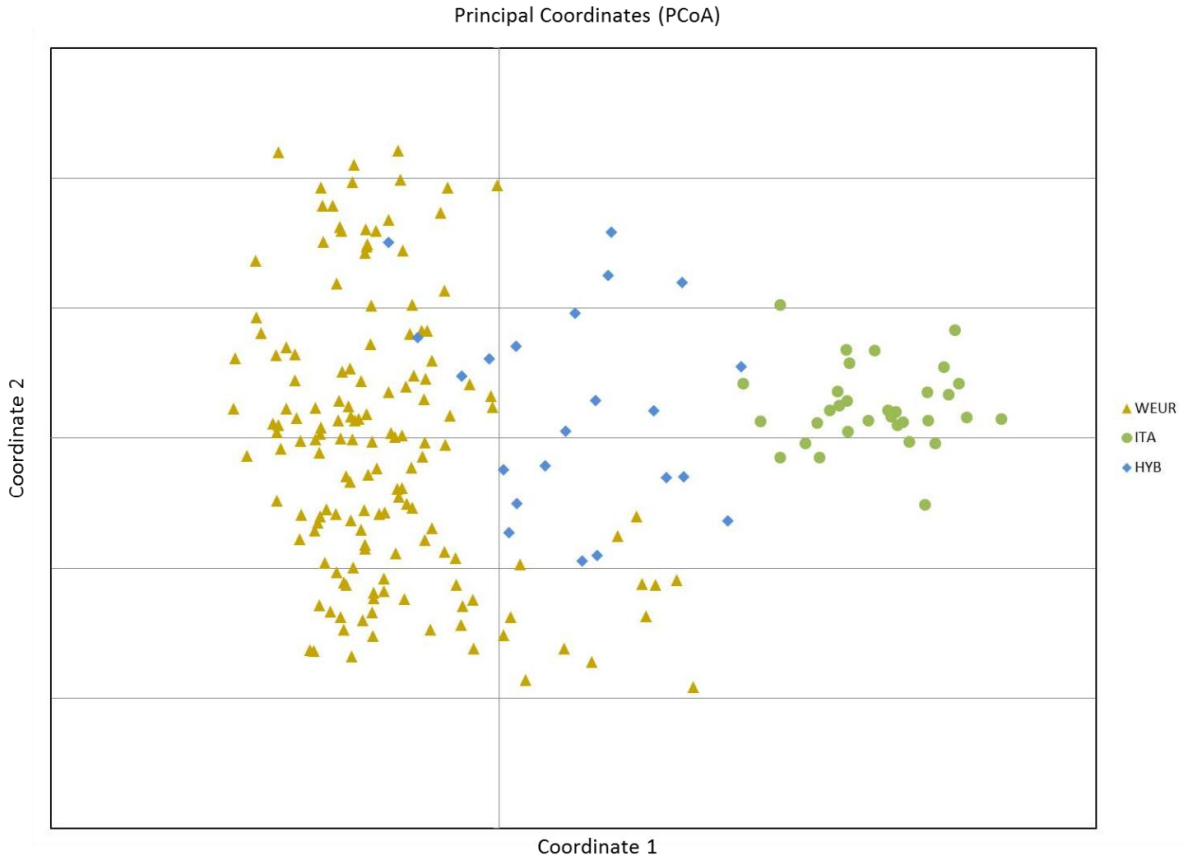
Supplementary Figure S2.6: Structure output microsatellite data for the introduced population in Holmsley, United Kingdom. Each individual is represented by a vertical line partitioned into K = 2 coloured segments according to the proportion of membership (%) in each cluster. Individual mtDNA haplotype lineage is indicated on the x-axis.



Supplementary Figure S2.7: Output from Principle Coordinate Analysis for animals from the non-native population in Holmsley, UK (blue squares). These animals were tested against four non-native populations of pure origin (three of Italian origin and one of Western European origin) that served as source populations (Michaelides et al. 2015).



Supplementary Figure S2.8: Structure output for the introduced population in Mannheim, Germany. Each individual is represented by a vertical line partitioned into K = 2 coloured segments according to the proportion of membership (%) in each cluster. Individual mtDNA haplotype is indicated on the x-axis.



Supplementary Figure S2.9: Output from Principle Coordinate Analysis for animals from the non-native population in Mannheim, Germany. Animals with Italian mtDNA haplotypes are represented by green circles. Animals with Western European mtDNA haplotypes are represented by yellow triangles.

2.9 Supplementary Tables

Supplementary Table S2.1: Data on all native (France and Italy) and non-native (UK and German) populations used in this study. This includes information on the geographical location of each population, the mitochondrial lineage each population belongs to (WEUR = Western European, ITA = Italian, Mixed = a mixture of genotypes from both lineages) and the sample sizes for the genetic and phenotypic analyses.

Country	Population	Lat	Long	MtDNA Lineage	Tot N.	SVL	Mass	Head L/W	Green Score	Black Score	Testes Mass	Bite Force
France	Dinan	48.45	-2.05	WEUR	25	25	25	24	25	24		3
France	Fonteirs-Cabardes	43.37	2.25	WEUR	20	20	20	20	20	20		
France	Josselin	47.95	-2.54	WEUR	25	25	25	25	25	25	14	15
France	Nebias	42.89	2.11	WEUR	26	26	25	26	26	25		
France	Pontchateau	47.43	-2.09	WEUR	25	25	25	25	25	25	18	21
France	Pouzagues	46.78	-0.84	WEUR	55	55	55	55	55	55	16	27
France	Puybelliard	46.71	-1.03	WEUR	19	19	19	19	19	18		
France	Saint-Gervais	46.90	-1.99	WEUR	29	28	28	28	28	28		
France	Saint-Lizier	43.00	1.13	WEUR	21	21	21	21	21	21		
France	Saint-Michel	46.35	-1.25	WEUR	24	23	23	23	23	20		
France	Vitre	48.12	-1.21	WEUR	20	20	20	20	20	20		
Germany	Mannheim	49.5	8.46	MIXED	203	0	0	0	0	0		

Italy	Calci	43.73	10.51	ITA	23	23	23	23	23	23		
Italy	Castelfioentino	43.61	10.97	ITA	12	12	12	12	12	11		
Italy	Castelnuovo Berardenga	43.35	11.51	ITA	9	9	9	9	9	9		
Italy	Certaldo	43.55	11.04	ITA	19	19	19	19	19	8		
Italy	Chianni	43.48	10.64	ITA	22	22	22	22	22	22		
Italy	Colle di Val D'Elsa	43.42	11.11	ITA	47	47	47	47	46	38	12	21
Italy	Crespina	43.57	10.56	ITA	21	21	21	21	21	20		
Italy	Greve in Chianti	43.59	11.31	ITA	57	57	57	57	57	52	10	20
Italy	Montemassi	42.99	11.06	ITA	22	22	22	22	22	22		
Italy	Peccoli	43.54	10.72	ITA	26	26	26	26	26	19		
Italy	Prato	43.88	11.10	ITA	30	30	30	30	30	30	10	17
Italy	Travale	43.17	11.01	ITA	22	22	22	22	22	22		
Italy	Buti	43.73	10.58	MIXED	29	29	28	29	29	29		
Italy	Gragnola	44.19	10.11	WEUR	22	22	22	22	22	21		
Italy	Levanto	44.17	9.61	WEUR	28	28	28	28	28	28		
Italy	Rapallo	44.35	9.23	WEUR	24	24	24	24	23	24		
Italy	Roccatagliata	44.47	9.20	WEUR	25	25	25	24	25	24		
Italy	San Bernardo	44.39	8.47	WEUR	9	9	9	9	9	9		

Italy	San Terenzo	44.08	9.89	WEUR	25	27	27	27	27	27		
Italy	Sesta Godano	44.29	9.67	WEUR	27	27	27	27	27	27		
Italy	Uscio	44.41	9.15	WEUR	27	27	27	27	27	27		
Italy	Viarreggio	43.86	10.25	MIXED	28	28	28	28	28	28		
Italy	Bardi	44.63	9.73	WEUR	28	28	28	28	28	28		
Italy	Brignano-Frascata	44.81	9.04	WEUR	26	26	26	26	26	26		
Italy	Cantalupo	44.86	8.55	WEUR	24	24	24	24	24	24	14	9
Italy	Loano	44.13	8.26	WEUR	12	12	12	12	12	12		
Italy	Mele	44.44	8.75	WEUR	28	28	28	28	28	28		
Italy	Noli	44.21	8.41	WEUR	27	27	27	27	27	27		
Italy	Pellegrino Parmesne	44.73	9.93	WEUR	27	27	27	27	27	27		
Italy	Perino	44.82	9.50	WEUR	25	25	25	25	25	25		
Italy	San Martino	44.39	8.52	WEUR	29	29	29	29	29	28		
Italy	Sassello	44.48	8.49	WEUR	26	26	26	26	26	26		
Italy	Silvano d'Orba	44.69	8.67	WEUR	25	25	25	25	25	25		
Italy	Valmozzola	44.58	9.94	WEUR	24	24	24	24	24	24		
Italy	Varazze	44.36	8.58	WEUR	38	38	38	38	38	38		
UK	Dancing Ledge	50.35	-2.04	ITA	58	58	15	56	58	57		
UK	Shoreham	50.49	-0.15	ITA	43	43	20	43	43	40		

UK	Ventnor Botanics	50.58	-1.22	ITA	34	34	25	34	30	28
UK	Ventnor Town	50.59	-1.21	ITA	59	59	43	59	59	57
UK	Winspit	50.35	-2.15	ITA	31	31	16	31	31	29
UK	Abbotsbury	50.66	-2.60	ITA	33	32	26	32	32	32
UK	Holmsley	50.47	-1.40	MIXED	27	27	9	27	27	27
UK	Poole	50.42	-1.55	ITA	51	50	0	50	50	49
UK	Bury	50.90	-0.56	WEUR	9	9	0	9	9	9
UK	Cheyne Wear	50.53	-2.43	WEUR	47	45	24	45	44	45
UK	Cheyne Wear North	50.54	-2.42	WEUR	39	39	21	39	39	39
UK	Wellington	50.97	-3.22	WEUR	25	25	11	25	24	22
UK	Wembdon	51.13	-3.02	WEUR	13	13	10	13	13	13

Supplementary Table S2.2: Ethogram detailing the behaviours recorded in the simulated secondary contact experiments (2010 and 2013) (Heathcote 2013).

Code	Behavour
1	Bask/Stationary (flattened)
2	Approach
3	Charge
4	Display (throat or outer ventral scales)
5	Alert-Forebody Raised
6	Attack
7	Head Grasp
8	Chase/Follow
9	Retreat
10	Freeze
11	Wave (in sight of another lizard)
12	Tail Quiver
13	Tail Grab
14	Male tongue flick on female
15	Mating
16	Female back pat male
17	Defecate
18	Moving/Patrolling
19	Male alert by Female
20	Fight
21	Hunting/Feeding
22	Male-Female Lying Together

Supplementary Table S2.3: Details for the twenty three microsattelite loci used in the study. Multiplexes 1, 2 and 3 were developed by Heathcote *et al.* (2015) and multiplex 4 and 5 were developed by Richard *et al.* (2012). Multiplex 1, 2 and 3 were used for paternity analysis in 2010, and 1 and 2 for paternity analysis in 2013. Multiplex 6, 7 and 8 were only used for Mannheim individuals and developed by US for primers published by Boudjemadi *et al.* (1999), Nembrini & Oppliger (2003) and Pinho *et al.* (2004).

Multiplex	Locus		Primer sequence (5'-3')	Repeat motif	Range (bp)
1	PmurC150	F	[6-FAM]GTCAGCTTTGCAGCACCTTAG	CA	171-217
		R	GCGATTAGAGAAGGCGTTTG		
	PmurC168	F	[HEX]GGTCCGGCTTCAAAGAATAAG	TTTC	210-306
		R	CAGAGGACTCGCTCAAGGAC		
	PmurC275_278	F	[6-FAM]GCTTAAAATTAATGCTGCTATTGTATC	TATC	219-610
		R	ATAGGTAGAAAATTTATAAACCCTTGG		
2	PmurC164	F	[6-FAM]ATCGATGAATGAAGGGCAGT	GATA	170-246
		R	CCAGGCATTGTCAAACTATCTG		
	PmurC038	F	[HEX]CAATGTGCAGTGTTGGGTTG	TATC	193-425
		R	ATGTGAGCGACTCCTGGATG		
	PmurC028	F	[6-FAM]TTGCTTCTGAATACGCCTAGC	TATC	253-543
		R	AGTGTATTGCGACTGTCAATGG		
3	PmurC356	F	[6-FAM]GATCTTCAGATGAAGGGTAGTTAGAT	GTTA	138-178
		R	ATGAAGACAAACAGGCTTGG		
	PmurC109	F	[HEX]AGGAGCCCAGCAGCTGAA	GTA	295-355
		R	TTTACATAGACCTGCGGGTATGG		
	PmurC103	F	[6-FAM]CCAGGTCTTGTGATCGAGTG	GATA	316-480
4	Pm01	F	[6-FAM] CCACAGGCATCTGGTTAG	$(ATT)_{16}$	119-137
		R	TCCATAAGACTGTAAGACAAGCC		
	Pm05	F	[HEX] CAAGAGGGCAGCCTAGTAATG	$(AGAT)_{10}$	135-185
		R	AGATGGGCTCATTTCAACTCC		
	Pm09	F	[NED] ACGTGTTTCTGTGCTTTGC	$(ATT)_{17}$	176-203
		R	AGTCAGACGAGAGGTTGCC		
	Pm16	F	[6-FAM] GGGATGGAGAAAGATGGCG	$(TCTT)_{16}$	179-211
		R	GCACTTGCCTACTGGTCATAC		
5	Pm02	F	[HEX] TTGGGAAGAAGGGGAAGGG	$(AACC)_7$	164-216
		R	ATGGCCGCTAGGTCAAGTG		
	Pm19	F	[6-FAM] CAGCCACAAGGTGAACCAG	$(AGGC)_{11}$	164-204
		R	TGTGAGGTCAGAGGCATGG		
	Pm14	F	[NED] GCAGGATCAGAGCGCAATC	$(GCAG)_7$	151-187
		R	TGTGGCATGTTGAGACACC		
6	C9	F: [HEX]CATTGCTGGTTCTGGAGAAAG		(CAA)3	122-154
		R: CCTGATGAAGGGAAGTGGTG			
	B4	F: [FAM]AATCTGCAATTCTGGGATGC		(AG)16	127-179

		R: AGAAGCAGGGGATGCTACAG		
	Pb73	F: [TAMRA]GCCCATGTCACTTCAGGTAGAAGC R:GAAAACTAGGAGTTAGGGAGAAGG	(CA)nCT(CA)n	243-397
7	Pb50	F: [FAM]GGATGTTTCAGCATGCTTGG R: AGACCTCACTGGGCCATTAC	(CA)n	92-104
	Lv472	F: [HEX]CCCTACTTGAGTTGCCGTC R: CTTTGCAGGTAACAGAGTAG	(AC)18	103-125
8	Pb10	F: [TAMRA]AGTGGAATCGGCTGCAATAC R: ACCAGTCCCAGGAATTTAGG	(CA)n	187-341
	Lv319	F: [FAM]TGTTGCTATTTTGTATGCTTAC R: CCTGTGACTGTCCTCAGAGG	(AC)22	144-194

Supplementary Table S2.4: Output from models testing for divergence in morphological and colour phenotypes between Western European and Italian lizards. Statistically significant P-values are in bold. Where the interaction is non-significant, main effects are reported from the model excluding the interaction. Non-parametric analyses of colour traits were run separately for lineage and sex (F = Female, M = Male, WE = Western European, IT = Italian).

	Trait	Lineage	Sex	Lineage*Sex	Covariate
Morphology	Snout-to-Vent Length (mm)	**$\chi2 = 5.66$, $p = 0.017$**	**$\chi2 = 4.75$, $p = 0.029$**	$\chi2 = 3.52$, $p = 0.060$	
	Head Length (mm)	**$\chi2 = 50.13$, $p < 0.001$**	**$\chi2 = 3100.46$, $p < 0.001$**	$\chi2 = 1.12$, $p = 0.289$	**SVL: $\chi2 = 2031.42$, $p < 0.001$**
	Bite Force	$\chi2 = 0.05$, $p = 0.815$	**$\chi2 = 15.38$, $p = 0.001$**	**$\chi2 = 15.28$, $p < 0.001$**	**HL: $\chi2 = 309.06$, $p < 0.001$**
	Testes Mass (g)	**$\chi2 = 12.03$, $p < 0.001$**			**SVL: $\chi2 = 38.32$, $p < 0.001$**
Colour	Greenness	**M: $\chi2 = 313.69$, $p < 0.001$**	**WE: $\chi2 = 53.91$, $p < 0.001$**		
	Greenness	**F: $\chi2 = 257.35$, $p < 0.001$**	**IT: $\chi2 = 17.56$, $p < 0.001$**		
	Blackness	**M: $\chi2 = 211.22$, $p < 0.001$**	**WE: $\chi2 = 79.80$, $p < 0.001$**		
	Blackness	**F: $\chi2 = 153.20$, $p < 0.001$**	**IT: $\chi2 = 84.31$, $p < 0.001$**		

Supplementary Table S2.5: Univariate and multivariate linear regression outputs for the relationship between dominance and three male phenotypic traits, head length, dorsal greenness and ventral blackness (standardized to a mean of 1 and a standard deviation of 0) across both experiments (2010 and 2013). Boldface indicates significance. Overall fit of the multivariate model was significant ($F_{3,99}$ = 23.97, P <0.001).

Analysis	Trait	β_i	95% CI
Multivariate	**Head Length**	**0.22**	**0.04, 0.40**
	Greenness	**0.37**	**0.11, 0.63**
	Blackness	0.11	-0.14, 0.37
Univariate	**Head Length**	**0.49**	**0.33, 0.65**
	Greenness	**0.59**	**0.44, 0.74**
	Blackness	**0.53**	**0.37, 0.69**

Supplementary Table S2.6: Output from models examining the differences between Western European and Italian wall lizards in the number of courtships males initiated, the number of offspring males sired and the proportion of a females clutch that were hybrids. Data comes from experimental secondary contact zone experiments carried out using non-native animals (2010) and native animals (2013) respectively. Statistically significant P-values are in bold.

	Non-Native Animals (2010)		**Native Animals (2013)**	
Trait	**Lineage**	**SVL**	**Lineage**	**SVL**
Number of Courtships	**$\chi2 = 6.98$, p = 0.008**	$\chi2 = 1.30$, p = 0.255	**$\chi2 = 52.44$, p < 0.001**	**$\chi2 = 8.55$, p = 0.003**
Number of Offspring	**$\chi2 = 4.48$, p = 0.034**	**$\chi2 = 4.51$, p = 0.033**	**$\chi2 = 5.39$, p = 0.020**	**$\chi2 = 4.56$, p = 0.033**
Proportion of Hybrid Offspring	**$\chi2 = 6.57$, p = 0.010**	$\chi2 = 1.14$, p = 0.285	**$\chi2 = 12.92$, p < 0.001**	$\chi2 = 0.12$, p = 0.733

Supplementary Table S2.7: Output from generalized linear mixed models examining the effect of dominance on the number of offspring a male sired.

	Non-Native Animals (2010)			**Native Animals (2013)**		
Trait	**Lineage**	**Dominance**	**SVL**	**Lineage**	**Dominance**	**SVL**
Number of Offspring	$\chi2 = 0.76$, $p = 0.38$	**$\chi2 = 12.25$, $p < 0.001$**	$\chi2 = 0.01$, $p = 0.95$	$\chi2 = 0.01$, $p = 0.89$	**$\chi2 = 4.98$, $p = 0.03$**	$\chi2 = 1.60$, $p = 0.24$

Supplementary Table S2.8: AICc for genetic and phenotypic cline models (Model I = fixed, no tails, Model II = free, no tails, Model III = fixed both tails, Model IV = free, both tails, Model V = fixed, left only tail, Model VI = free, left only tail, Model VII = fixed, right only tail, Model VIII = free, right only tail, Model IX = fixed, mirror tail, Model X = free, mirror tail).

Cline variable	Model I	Model II	Model III	Model IV	Model V	Model VI	Model VII	Model VIII	Model IX	Model X
mtDNA	**43.3**	47.6	47.4	56.3	47.5	51.8	43.8	51.8	47.5	51.8
Hybrid Index	19.5	21.6	16.6	20.2	23.4	25.7	**12.9**	16.2	16.8	16.8
Greenness (M)	381.4	**370.9**	388.8	379.5	384.8	374.7	386.6	375.3	386.5	375.5
Greenness (F)	336.2	**283.5**	304.1	297.8	299.7	304.9	340	303.2	339.9	290.6
Blackness (M)	-154.8	**-157**	-146.4	-147.5	-150.6	-153.3	-150.6	-152.9	-150.6	-153
Blackness (F)	-129.4	-145.4	-121	-130.8	-125.3	-143.9	-125.3	**-152.5**	-125.3	-151.2
Head length (M)	**497.7**	500.8	505.9	509.2	501.8	508.4	501.7	504.2	501.8	506.2
Headlength (F)	**364.1**	365.8	372	391.1	368.2	369.9	367.1	368.9	368.2	369.1

Supplementary Table S2.9: Output from the computer program New Hybrid indicating the level of genetic admixture with individuals from the UK population at Homsley as well as those from four representative pure populations. The program gives the probability that each individual (sample) belongs to any of the different categories; Pure (parental population 1 or 2), F1 (first generation individual), F2 (second generation individual), BX1 (backcross between an F1 and Pure 1 individual), BX2 (backcross between an F1 and Pure 2 individual). All individuals from Holmsley were identified as being pure Italian despite four individuals having mtDNA haplotypes from Western Europe (hybrid individual's bolded).

Sample	MtDNA Lineage	PURE1	PURE2	F1	F2	BX1	BX2
WEW03	WEUR	0	0.99993	0	0.00001	0	0.00006
WEW04	WEUR	0	1	0	0	0	0
WEW05	WEUR	0	0.99969	0	0.00001	0	0.00029
WEW06	WEUR	0	1	0	0	0	0
WEW07	WEUR	0	0.99999	0	0	0	0.00001
WEW08	WEUR	0	1	0	0	0	0
WEW09	WEUR	0	0.99998	0	0	0	0.00002
WEW10	WEUR	0	1	0	0	0	0
WEW14	WEUR	0	0.99999	0	0	0	0.00001
WEW28	WEUR	0	0.99998	0	0	0	0.00002
WEW29	WEUR	0	0.99999	0	0	0	0.00001
WEW30	WEUR	0	1	0	0	0	0
WEW31	WEUR	0	0.99915	0	0.00003	0	0.00082
WEW32	WEUR	0	0.99999	0	0	0	0.00001
WEW33	WEUR	0	0.99999	0	0	0	0.00001
WEW34	WEUR	0	0.99964	0	0	0	0.00036
WEW36	WEUR	0	0.99998	0	0	0	0.00002
WEW37	WEUR	0	0.99998	0	0	0	0.00002
WEW38	WEUR	0	0.99999	0	0	0	0.00001
WEW39	WEUR	0	0.99997	0	0	0	0.00003
WEW40	WEUR	0	0.99989	0	0	0	0.00011
WEW41	WEUR	0	1	0	0	0	0
WEW42	WEUR	0	1	0	0	0	0
WEW45	WEUR	0	1	0	0	0	0
WEW46	WEUR	0	1	0	0	0	0
VTV01	ITA	0.99999	0	0	0	0.00001	0
VTV02	ITA	0.99998	0	0	0	0.00002	0
VTV03	ITA	1	0	0	0	0	0
VTV10	ITA	1	0	0	0	0	0
VTV11	ITA	0.99993	0	0	0	0.00007	0
VTV13	ITA	0.99997	0	0	0	0.00003	0
VTV14	ITA	0.99995	0	0	0	0.00005	0
VTV15	ITA	1	0	0	0	0	0
VTV16	ITA	0.99998	0	0	0	0.00002	0
VTV17	ITA	1	0	0	0	0	0
VTV19	ITA	0.99999	0	0	0	0.00001	0

VTV20	ITA	1	0	0	0	0	0
VTV22	ITA	0.99997	0	0	0	0.00003	0
VTV24	ITA	1	0	0	0	0	0
VTV25	ITA	1	0	0	0	0	0
VTV26	ITA	0.99998	0	0	0	0.00002	0
VTV28	ITA	1	0	0	0	0	0
VTV31	ITA	0.99998	0	0	0	0.00002	0
VTV35	ITA	1	0	0	0	0	0
VTV38	ITA	0.99999	0	0	0	0.00001	0
VTV41	ITA	1	0	0	0	0	0
VTV43	ITA	1	0	0	0	0	0
VTV44	ITA	0.99996	0	0	0	0.00004	0
VTV51	ITA	1	0	0	0	0	0
BB01	ITA	0.99979	0	0	0	0.00021	0
BB02	ITA	0.99974	0	0	0	0.00026	0
BB03	ITA	0.99998	0	0	0	0.00002	0
BB04	ITA	0.99978	0	0	0	0.00022	0
BB05	ITA	0.99992	0	0	0	0.00008	0
BB06	ITA	0.99999	0	0	0	0.00001	0
BB07	ITA	0.99972	0	0	0	0.00027	0
BB08	ITA	0.40608	0	0	0.03622	0.5577	0
BB09	ITA	0.99982	0	0	0	0.00018	0
BB10	ITA	0.99999	0	0	0	0.00001	0
BB11	ITA	0.99999	0	0	0	0.00001	0
BB12	ITA	0.99999	0	0	0	0.00001	0
BB13	ITA	0.99999	0	0	0	0.00001	0
SH000stump	ITA	0.99998	0	0	0	0.00002	0
SH004/51/3/5	ITA	1	0	0	0	0	0
SH0150	ITA	0.99989	0	0	0	0.00011	0
SH0203	ITA	0.9997	0	0	0	0.0003	0
SH0205	ITA	0.99999	0	0	0	0.00001	0
SH0210	ITA	0.99956	0	0	0	0.00044	0
SH0215	ITA	1	0	0	0	0	0
SH0225	ITA	1	0	0	0	0	0
SH0232	ITA	0.99999	0	0	0	0.00001	0
SH0250	ITA	0.99999	0	0	0	0.00001	0
SH0251	ITA	0.99995	0	0	0	0.00005	0
SH0252	ITA	0.99995	0	0	0	0.00005	0
SH0253	ITA	0.99999	0	0	0	0.00001	0
SH0302	ITA	0.99999	0	0	0	0.00001	0
SH0303	ITA	0.99433	0	0	0.00003	0.00563	0
SH0305	ITA	1	0	0	0	0	0
SH0310	ITA	0.99999	0	0	0	0.00001	0
SH0311	ITA	0.99999	0	0	0	0.00001	0
SH0313	ITA	1	0	0	0	0	0
SH0321	ITA	0.99999	0	0	0	0.00001	0

SH0322	ITA	0.99897	0	0	0	0.00103	0
SH0323	ITA	0.99983	0	0	0	0.00017	0
SH1004	ITA	0.99997	0	0	0	0.00003	0
SH1005	ITA	0.99998	0	0	0	0.00002	0
SH1010	ITA	0.99997	0	0	0	0.00003	0
HO01	ITA	0.99995	0	0	0	0.00005	0
HO02	ITA	0.99984	0	0	0	0.00016	0
HO03	ITA	0.99847	0	0	0.00007	0.00147	0
HO04	ITA	0.9989	0	0	0	0.00109	0
HO05	ITA	0.99999	0	0	0	0.00001	0
HO06	ITA	0.91413	0	0	0.00101	0.08486	0
HO07	**WEUR**	**0.99345**	**0**	**0**	**0.00014**	**0.00641**	**0**
HO08	ITA	0.99995	0	0	0	0.00005	0
HO09	ITA	0.99996	0	0	0	0.00004	0
HO10	ITA	0.99997	0	0	0	0.00003	0
HO11	**WEUR**	**0.94451**	**0**	**0**	**0.00027**	**0.05522**	**0**
HO12	ITA	0.99339	0	0	0.00005	0.00657	0
HO13	ITA	0.99999	0	0	0	0.00001	0
HO14	ITA	0.99804	0	0	0	0.00196	0
HO15	ITA	0.99999	0	0	0	0.00001	0
HO18	**WEUR**	**0.99999**	**0**	**0**	**0**	**0.00001**	**0**
HO19	ITA	0.99999	0	0	0	0.00001	0
HO20	ITA	0.99997	0	0	0	0.00003	0
HO21	ITA	0.99997	0	0	0	0.00003	0
HO22	ITA	0.99996	0	0	0	0.00004	0
HO23	ITA	0.99995	0	0	0	0.00005	0
HO24	ITA	0.99995	0	0	0	0.00005	0
HO25	ITA	0.99996	0	0	0	0.00004	0
HO26	ITA	0.99999	0	0	0	0.00001	0
HO27	ITA	0.99835	0	0	0.00001	0.00164	0
HO17	**WEUR**	**0.99551**	**0**	**0**	**0.00026**	**0.00423**	**0**

Supplementary Table S2.10: Output from the computer program New Hybrid indicating the level of genetic admixture with individuals from the German population at Mannheim. The program gives the probability that each individual (sample) belongs to any of the different categories; Pure (parental population 1 or 2), F1 (first generation individual), F2 (second generation individual), BX1 (backcross between an F1 and Pure 1 individual), BX2 (backcross between an F1 and Pure 2 individual). Eight hybrid individuals in Mannheim harbored Italian mtDNA haplotypes and the rest (15) had mtDNA haplotypes from Western Europe (hybrid individuals in bold).

Sample	MtDNALineage	PURE1	PURE2	F1	F2	BX1	BX2
Ma_152	**ITA**	**0**	**0**	**0.00001**	**0.99907**	**0.00007**	**0.00086**
Ma_157	**ITA**	**0**	**0**	**0.00001**	**0.99847**	**0.00052**	**0.001**
Ma_158	**ITA**	**0**	**0**	**0**	**0.99754**	**0.00246**	**0**
Ma_160	**ITA**	**0**	**0.00018**	**0**	**0.99751**	**0**	**0.00231**
Ma_131	**ITA**	**0**	**0**	**0**	**0.98786**	**0**	**0.01213**
Ma_007	**ITA**	**0.00007**	**0**	**0**	**0.97504**	**0.02489**	**0**
Ma_161	**ITA**	**0.0104**	**0**	**0**	**0.87552**	**0.11407**	**0**
Ma_159	**ITA**	**0**	**0.00037**	**0**	**0.5515**	**0**	**0.44814**
Ma_033	ITA	0.88267	0	0	0.06707	0.05026	0
Ma_025	ITA	0.9076	0	0	0.02995	0.06245	0
Ma_120	ITA	0.96953	0	0	0.01497	0.0155	0
Ma_044	ITA	0.99223	0	0	0.00328	0.00449	0
Ma_185	ITA	0.99844	0	0	0.00066	0.0009	0
Ma_187	ITA	0.99851	0	0	0.00036	0.00113	0
Ma_188	ITA	0.99903	0	0	0.00023	0.00074	0
Ma_045	ITA	0.99942	0	0	0.00016	0.00041	0
Ma_042	ITA	0.99965	0	0	0.00013	0.00023	0
Ma_030	ITA	0.99953	0	0	0.00007	0.0004	0
Ma_036	ITA	0.99968	0	0	0.00007	0.00026	0
Ma_032	ITA	0.99975	0	0	0.00006	0.00019	0
Ma_028	ITA	0.99978	0	0	0.00005	0.00017	0
Ma_035	ITA	0.99975	0	0	0.00005	0.0002	0
Ma_039	ITA	0.99974	0	0	0.00005	0.00021	0
Ma_041	ITA	0.99981	0	0	0.00005	0.00014	0
Ma_038	ITA	0.99981	0	0	0.00004	0.00015	0
Ma_163	ITA	0.99977	0	0	0.00004	0.00019	0
Ma_162	ITA	0.99987	0	0	0.00003	0.0001	0
Ma_164	ITA	0.99976	0	0	0.00003	0.0002	0
Ma_027	ITA	0.99984	0	0	0.00002	0.00014	0
Ma_024	ITA	0.99992	0	0	0.00001	0.00007	0
Ma_029	ITA	0.99992	0	0	0.00001	0.00008	0
Ma_034	ITA	0.99992	0	0	0.00001	0.00007	0
Ma_040	ITA	0.99991	0	0	0.00001	0.00008	0
Ma_022	ITA	0.99999	0	0	0	0.00001	0
Ma_023	ITA	0.99997	0	0	0	0.00003	0
Ma_026	ITA	0.99997	0	0	0	0.00003	0

Ma_031	ITA	0.99996	0	0	0	0.00003	0
Ma_037	ITA	0.99993	0	0	0	0.00007	0
Ma_043	ITA	0.99995	0	0	0	0.00004	0
Ma_186	ITA	0.99997	0	0	0	0.00003	0
Ma_005	**WEUR**	**0**	**0.00756**	**0**	**0.96921**	**0**	**0.02322**
Ma_119	**WEUR**	**0**	**0**	**0**	**0.9618**	**0**	**0.03819**
Ma_008	**WEUR**	**0**	**0.00018**	**0**	**0.90548**	**0**	**0.09433**
Ma_009	**WEUR**	**0**	**0.0005**	**0.00014**	**0.77925**	**0.00001**	**0.2201**
Ma_139	WEUR	0	0.90068	0	0.07095	0	0.02837
Ma_141	WEUR	0	0.84892	0	0.04672	0	0.10436
Ma_143	WEUR	0	0.99322	0	0.00219	0	0.00459
Ma_013	WEUR	0	0.99544	0	0.00087	0	0.00369
Ma_147	WEUR	0	0.99734	0	0.00038	0	0.00228
Ma_142	WEUR	0	0.9895	0	0.00037	0	0.01013
Ma_114	WEUR	0	0.99929	0	0.00007	0	0.00064
Ma_138	WEUR	0	0.99963	0	0.00004	0	0.00033
Ma_181	WEUR	0	0.99966	0	0.00003	0	0.0003
Ma_054	WEUR	0	0.9996	0	0.00002	0	0.00038
Ma_156	WEUR	0	0.99962	0	0.00001	0	0.00037
Ma_179	WEUR	0	0.99955	0	0.00001	0	0.00044
Ma_084	WEUR	0	0.99994	0	0	0	0.00005
Ma_118	WEUR	0	0.99991	0	0	0	0.00009
Ma_183	WEUR	0	0.99986	0	0	0	0.00013
Ma_121	**WEUR**	**0**	**0.06323**	**0**	**0.92906**	**0**	**0.00771**
Ma_129	**WEUR**	**0**	**0.05267**	**0**	**0.73191**	**0**	**0.21542**
Ma_176	**WEUR**	**0**	**0.00002**	**0**	**0.71362**	**0**	**0.28636**
Ma_050	**WEUR**	**0**	**0.29654**	**0**	**0.65154**	**0**	**0.05191**
Ma_145	**WEUR**	**0**	**0.39853**	**0**	**0.34387**	**0**	**0.2576**
Ma_116	WEUR	0	0.65573	0	0.05588	0	0.28839
Ma_083	WEUR	0	0.76134	0	0.02359	0	0.21507
Ma_085	WEUR	0	0.83734	0	0.01734	0	0.14532
Ma_133	WEUR	0	0.88122	0	0.01516	0	0.10362
Ma_192	WEUR	0	0.9827	0	0.01267	0	0.00464
Ma_140	WEUR	0	0.98323	0	0.00571	0	0.01107
Ma_080	WEUR	0	0.94375	0	0.00359	0	0.05266
Ma_062	WEUR	0	0.92707	0	0.0031	0	0.06983
Ma_105	WEUR	0	0.99089	0	0.00229	0	0.00683
Ma_165	WEUR	0	0.99563	0	0.00186	0	0.00251
Ma_107	WEUR	0	0.98922	0	0.00156	0	0.00922
Ma_051	WEUR	0	0.99185	0	0.00131	0	0.00683
Ma_100	WEUR	0	0.99406	0	0.00117	0	0.00477
Ma_068	WEUR	0	0.9978	0	0.00087	0	0.00133
Ma_180	WEUR	0	0.99527	0	0.0007	0	0.00402
Ma_115	WEUR	0	0.98502	0	0.00056	0	0.01442
Ma_072	WEUR	0	0.99606	0	0.00047	0	0.00347
Ma_091	WEUR	0	0.99608	0	0.00037	0	0.00356

Ma_057	WEUR	0	0.99743	0	0.00025	0	0.00232
Ma_190	WEUR	0	0.99861	0	0.00019	0	0.0012
Ma_052	WEUR	0	0.99928	0	0.00014	0	0.00058
Ma_061	WEUR	0	0.99864	0	0.00012	0	0.00124
Ma_132	WEUR	0	0.99864	0	0.00012	0	0.00124
Ma_110	WEUR	0	0.99749	0	0.0001	0	0.00241
Ma_011	WEUR	0	0.99924	0	0.00009	0	0.00067
Ma_059	WEUR	0	0.99909	0	0.00007	0	0.00084
Ma_125	WEUR	0	0.99898	0	0.00007	0	0.00095
Ma_019	WEUR	0	0.99954	0	0.00006	0	0.00041
Ma_048	WEUR	0	0.99938	0	0.00006	0	0.00055
Ma_017	WEUR	0	0.99959	0	0.00004	0	0.00037
Ma_094	WEUR	0	0.99907	0	0.00004	0	0.0009
Ma_197	WEUR	0	0.99935	0	0.00004	0	0.0006
Ma_047	WEUR	0	0.99948	0	0.00003	0	0.00049
Ma_070	WEUR	0	0.99964	0	0.00003	0	0.00034
Ma_128	WEUR	0	0.99964	0	0.00003	0	0.00033
Ma_174	WEUR	0	0.99957	0	0.00003	0	0.0004
Ma_006	WEUR	0	0.99939	0	0.00002	0	0.00059
Ma_069	WEUR	0	0.99973	0	0.00002	0	0.00025
Ma_074	WEUR	0	0.99966	0	0.00002	0	0.00032
Ma_075	WEUR	0	0.99953	0	0.00002	0	0.00045
Ma_079	WEUR	0	0.99918	0	0.00002	0	0.0008
Ma_093	WEUR	0	0.99975	0	0.00002	0	0.00023
Ma_095	WEUR	0	0.99946	0	0.00002	0	0.00053
Ma_127	WEUR	0	0.99956	0	0.00002	0	0.00042
Ma_134	WEUR	0	0.99966	0	0.00002	0	0.00032
Ma_136	WEUR	0	0.99958	0	0.00002	0	0.0004
Ma_178	WEUR	0	0.99969	0	0.00002	0	0.00029
Ma_184	WEUR	0	0.99944	0	0.00002	0	0.00053
Ma_203	WEUR	0	0.99977	0	0.00002	0	0.00021
Ma_015	WEUR	0	0.99984	0	0.00001	0	0.00016
Ma_018	WEUR	0	0.99972	0	0.00001	0	0.00027
Ma_021	WEUR	0	0.99986	0	0.00001	0	0.00013
Ma_055	WEUR	0	0.99986	0	0.00001	0	0.00013
Ma_056	WEUR	0	0.99956	0	0.00001	0	0.00043
Ma_071	WEUR	0	0.99962	0	0.00001	0	0.00037
Ma_073	WEUR	0	0.99979	0	0.00001	0	0.0002
Ma_098	WEUR	0	0.99982	0	0.00001	0	0.00018
Ma_101	WEUR	0	0.99968	0	0.00001	0	0.00031
Ma_102	WEUR	0	0.99976	0	0.00001	0	0.00023
Ma_103	WEUR	0	0.99975	0	0.00001	0	0.00023
Ma_109	WEUR	0	0.99951	0	0.00001	0	0.00049
Ma_122	WEUR	0	0.99975	0	0.00001	0	0.00025
Ma_123	WEUR	0	0.99971	0	0.00001	0	0.00028
Ma_130	WEUR	0	0.99977	0	0.00001	0	0.00022

Ma_135	WEUR	0	0.9997	0	0.00001	0	0.0003
Ma_137	WEUR	0	0.99961	0	0.00001	0	0.00038
Ma_149	WEUR	0	0.99981	0	0.00001	0	0.00018
Ma_169	WEUR	0	0.99981	0	0.00001	0	0.00018
Ma_193	WEUR	0	0.99981	0	0.00001	0	0.00018
Ma_201	WEUR	0	0.99984	0	0.00001	0	0.00015
Ma_001	WEUR	0	0.99993	0	0	0	0.00007
Ma_002	WEUR	0	0.99993	0	0	0	0.00007
Ma_003	WEUR	0	0.99999	0	0	0	0.00001
Ma_004	WEUR	0	0.99996	0	0	0	0.00004
Ma_010	WEUR	0	0.99994	0	0	0	0.00006
Ma_012	WEUR	0	0.99984	0	0	0	0.00015
Ma_014	WEUR	0	0.99988	0	0	0	0.00011
Ma_016	WEUR	0	0.99983	0	0	0	0.00016
Ma_020	WEUR	0	0.99996	0	0	0	0.00004
Ma_046	WEUR	0	0.99982	0	0	0	0.00018
Ma_049	WEUR	0	0.99963	0	0	0	0.00036
Ma_058	WEUR	0	0.99996	0	0	0	0.00004
Ma_060	WEUR	0	0.99987	0	0	0	0.00013
Ma_063	WEUR	0	0.9999	0	0	0	0.0001
Ma_064	WEUR	0	0.9999	0	0	0	0.0001
Ma_065	WEUR	0	0.99995	0	0	0	0.00005
Ma_066	WEUR	0	0.99991	0	0	0	0.00009
Ma_067	WEUR	0	0.99991	0	0	0	0.00009
Ma_076	WEUR	0	0.99989	0	0	0	0.0001
Ma_078	WEUR	0	0.99983	0	0	0	0.00017
Ma_081	WEUR	0	0.99995	0	0	0	0.00005
Ma_082	WEUR	0	0.99999	0	0	0	0.00001
Ma_086	WEUR	0	0.99998	0	0	0	0.00002
Ma_087	WEUR	0	0.99998	0	0	0	0.00002
Ma_088	WEUR	0	0.99985	0	0	0	0.00014
Ma_089	WEUR	0	0.99997	0	0	0	0.00003
Ma_090	WEUR	0	0.99998	0	0	0	0.00002
Ma_092	WEUR	0	0.99995	0	0	0	0.00005
Ma_096	WEUR	0	0.99994	0	0	0	0.00006
Ma_097	WEUR	0	0.99991	0	0	0	0.00009
Ma_099	WEUR	0	0.99988	0	0	0	0.00012
Ma_104	WEUR	0	0.99988	0	0	0	0.00012
Ma_106	WEUR	0	0.99996	0	0	0	0.00004
Ma_108	WEUR	0	0.9999	0	0	0	0.0001
Ma_111	WEUR	0	0.99994	0	0	0	0.00005
Ma_112	WEUR	0	0.99997	0	0	0	0.00002
Ma_113	WEUR	0	0.99995	0	0	0	0.00005
Ma_117	WEUR	0	0.99997	0	0	0	0.00003
Ma_124	WEUR	0	0.99992	0	0	0	0.00008
Ma_126	WEUR	0	0.99997	0	0	0	0.00003

Ma_148	WEUR	0	0.99994	0	0	0	0.00006
Ma_150	WEUR	0	0.99998	0	0	0	0.00002
Ma_151	WEUR	0	0.99995	0	0	0	0.00005
Ma_166	WEUR	0	0.99996	0	0	0	0.00004
Ma_167	WEUR	0	0.99978	0	0	0	0.00022
Ma_168	WEUR	0	0.99996	0	0	0	0.00004
Ma_170	WEUR	0	0.99974	0	0	0	0.00026
Ma_171	WEUR	0	0.9998	0	0	0	0.0002
Ma_172	WEUR	0	0.99997	0	0	0	0.00003
Ma_173	WEUR	0	0.9999	0	0	0	0.0001
Ma_175	WEUR	0	0.99998	0	0	0	0.00002
Ma_177	WEUR	0	0.99987	0	0	0	0.00013
Ma_182	WEUR	0	0.99997	0	0	0	0.00003
Ma_194	WEUR	0	0.99995	0	0	0	0.00005
Ma_195	WEUR	0	0.99996	0	0	0	0.00004
Ma_196	WEUR	0	0.99998	0	0	0	0.00002
Ma_198	WEUR	0	0.9998	0	0	0	0.00019
Ma_199	WEUR	0	0.99993	0	0	0	0.00007
Ma_200	WEUR	0	0.99997	0	0	0	0.00003
Ma_202	WEUR	0	0.99988	0	0	0	0.00012
Ma_191	**WEUR**	**0**	**0**	**0**	**0.99978**	**0.00021**	**0**
Ma_153	**WEUR**	**0**	**0**	**0.00001**	**0.9986**	**0.00086**	**0.00053**
Ma_189	**WEUR**	**0**	**0.00175**	**0**	**0.9814**	**0**	**0.01685**
Ma_146	**WEUR**	**0**	**0.00001**	**0**	**0.97781**	**0.00003**	**0.02214**
Ma_053	**WEUR**	**0**	**0.00255**	**0.00022**	**0.97605**	**0.00038**	**0.0208**
Ma_144	**WEUR**	**0**	**0.00356**	**0.00003**	**0.97439**	**0.02035**	**0.00167**
Ma_154	WEUR	0	0.89938	0	0.0552	0	0.04542
Ma_155	WEUR	0	0.9933	0	0.00131	0	0.00539
Ma_077	WEUR	0	0.99971	0	0.00001	0	0.00028

Supplementary Table S2.11: Output from generalized linear mixed models examining the effect of treatment on the number of courtships males initiated, the number of offspring males sired and the proportion of a females clutch that were hybrids from the 2010 experiment. SVL = snout-to-vent length.

	Non-Native Animals (2010)			
Trait	**Lineage**	**Treatment**	**Lineage*Treat**	**SVL**
Number of Courtships	**$\chi2 = 6.98, p < 0.01$**	$\chi2 = 0.01, p = 0.93$	$\chi2 = 0.12, p = 0.73$	$\chi2 = 1.28, p = 0.26$
Number of Offspring	**$\chi2 = 4.36, p = 0.04$**	$\chi2 = 1.19, p = 0.27$	$\chi2 = 0.33, p = 0.56$	**$\chi2 = 4.46, p = 0.03$**
Proportion of Hybrid Offspring	**$\chi2 = 6.84, p < 0.01$**	$\chi2 = 0.44, p = 0.51$	$\chi2 = 1.17, p = 0.28$	$\chi2 = 1.34, p = 0.25$

Supplementary Table S2.12: Correlations among dominance and male phenotypic traits based on Pearson's and Spearmans' Correlation Coefficients. Scores in the upper section of the matrix are for lizards from the non-native experiment in 2010. Scores in the lower section of the matrix are for lizards from the native experiment in 2013 experiment. P-values are in brackets.

	Dominance	**Head Length**	**Greenness**	**Blackness**
Dominance	x	0.39 (0.01)	0.38 (0.01)	0.50 (0.001)
Head Length	060 (< 0.001)	x	0.57 (< 0.001)	0.46 (< 0.001)
Greenness	0.74 (< 0.001)	0.52 (< 0.001)	x	0.81 (< 0.001)
Blackness	0.66 (< 0.001)	0.54 (< 0.001)	0.80 (< 0.001)	x

2.10 Supplementary Information

Additional information on the quantification of phenotypic traits

Colouration: Each lizard was scored for two diagnostic colour variables, dorsal greenness and ventral blackness. Upon capture each lizard was scored for dorsal greenness by eye (see below) and photographed dorsally and ventrally with a Canon EOS 350D digital camera (Canon U.S.A., Inc., Lake Success, NY) using an X-rite Colour-Checker chart as background (e.g., Robertson & Robertson 2008; Wang & Shaffer 2008).

i) Dorsal greenness: Upon capture, dorsal greenness was scored visually based on intensity of greenness on a scale of 1 to 10 (1 being pure brown, 10 being intensely green, see Figure S2.2 for representative photos of each score). Whenever possible, lizards were scored by two experimenters (GMW and TU). Consistency of scoring between experimenters was very high ($r = 0.98$, $n = 915$, $p < 0.001$). We also tested the extent to which these subjective dorsal greenness scores corresponded with more objective measures of greenness by quantifying greenness for a subset of animals using two objective colouration measures. First, for animals from the UK sampled in 2011 ($n = 144$) we quantified dorsal greenness from digital photographs by calculating the proportion of green pixels on the dorsal surface of each lizard and dividing the green pixel count by the total RGB pixel count for that area using Photoshop vC4. We controlled for differing lighting and photographic conditions across different photos by dividing the proportion of green pixels on the lizard with the proportion of green pixels on the green standard of the X-rite Colour-Checker chart. Second, for males from the 2013 experiment ($n = 55$) we calculated dorsal (green/brown) chroma using a USB-2000 portable Ocean Optics diode-array spectrometer and a PX-2 xenon strobe light source. We measured dorsal chroma as the proportion reflectance between wavelengths of green light of our sample of males (R_{496}-R_{570}/R_{300}-R_{700}). In both instances our subjective colour scores were highly correlated with the values obtained for the objective measures (digital photos: $r = 0.86$, $p < 0.001$,

spectrophotometer: r = 0.89, p < 0.001). As we have subjective scores for all lizards across all experiments we used these scores throughout. Although *Podarcis muralis* is able to perceive the near ultraviolet portion of the light spectrum (Pérez I de Lanuza & Font 2014), the dorsal coloration of this species does not especially reflect in the UV and a human visual system of colour categorization is justified.

ii) Ventral blackness: Ventral blackness was scored by quantifying the proportion of black to non-black pixels on each lizard's chest using the program ImageJ (available at http://imagej.nih.gov). The chest section (see Figure S2.1) was highlighted on each individual and the area of black pixels selected manually using the threshold function. This area was compared to the total area of the section selected. The chest was selected as a representative area of an individual's overall ventral surface as it was highly consistent with that of other areas of the ventral surface (throat: r = 0.89, P < 0.001, n = 44, belly: r = 0.92, P < 0.001, n = 44). Ventral scoring was undertaken by two experimenters (GMW and NZ). Consistency in scoring was high between experimenters (r = 0.97, p < 0.001, n = 48).

Bite Force: This character required animals (n = 133) to be brought back from the field to minimize confounding variation in handling and body temperature. Each animal was tested in the middle of the lab light cycle, to maximise the likelihood that they had reached their optimal body temperature and showed minimal variance in temperature. Bite force measures were collected using a specially designed bite force meter, constructed from a modified Sauter FK 25N force meter. Bite force was recorded by encouraging a lizard to bite on two metal 'biting plates' (with insulation tape attached so lizards did not damage their teeth). One of the plates was attached to the main body of the force meter, and the other attached to the recording rod, ensuring that the two plates had a maximum gap of 1mm. Lizards biting the two plates therefore allowed their bite force to be recorded by depressing the recording rod onto the meter. In all recordings the position and angle of the lizard's head was kept as similar as

possible in relation to the biting plates. Three maximum bite force recordings were taken from each lizard at 30-second intervals. We retained the largest maximum bite force recording as the representative measure for individual maximum bite force. To control for any variation in body temperature at the time of testing, we recorded the skin surface temperature of each individual using an infrared duel laser digital thermometer. Body temperature did not predict maximum bite force and it was subsequently dropped from all analyses ($\chi^2 = 1.31$, $p = 0.25$).

Testes Mass: This character required a subset of animals to be sacrificed (n = 94). All males were euthanized (using concussion followed by permanent destruction of the brain) and then dissected. Both testes were removed and weighed to the nearest 0.001 grams and their mean mass was retained for analyses.

Additional information on the enclosure experiments

In both experiments (2010 and 2013) we collected individuals from multiple populations (n = 10 and 7) in order to reduce population of origin effects. In 2010, we collected animals from the UK locations of Shoreham, West Worthing, Ventnor Town, Ventnor Botanics, Winspit, Poole (all pure Italian origin), Bury, East Portland, Cheyne Weare and Wellington (all pure Western European origin). In 2013, we collected animals from Prato, Greve in Chianti, Colle di Val D'Elsa (all pure Italian origin), Dinan, Josselin, Pontchateau and Pouzagues (all pure Western European origin) (see Table S2.1 for full population details). Each enclosure was fitted with suitable habitat (bricks, wooden pallets) and stocked with 16 animals, four males and four females of each lineage. The exception to this was three enclosures in 2010 that were stocked with five Italian females and three French due to limited sample size of French females. One enclosure was stocked with only one French female and was removed from further analysis. The densities and sex ratios are within the range found within native and non-native populations.

Both the experiments followed identical protocols in terms of data analysis but differed in the structure of the habitat available, as follows:

In the experiment using lizards from non-native populations (2010) we modified the spatial clustering of habitat. In five of the enclosures nine areas of suitable habitat (bricks, wooden pallets) were dispersed across each enclosure evenly, whereas in the other five they were clumped together in the middle of the enclosure. To test the extent to which these two treatments influenced the results in this paper we added treatment as a fixed factor to our models of dominance, the number of females courted, the number of offspring sired and the proportion of hybrid offspring within a females clutch. Treatment had a marginally significant effect on dominance (permutation test using QAP - lineage: $P = 0.020$, treatment: $P = 0.071$, snout-to-vent length: $P = 0.013$) but did not influence any of the other three variables (Table S2.11). In all cases lineage remained highly significant (Table S2.11). We therefore do not consider treatment effects further in this paper. In the experiment using lizards from native populations (2013) all enclosures consisted of the same quantity and quality of suitable habitats.

Additional information on Behavioural Data Collection

Behavioural interaction data were obtained from 45 minute observation periods per enclosure conducted by three (in 2010) or two (in 2013) observers. Observations started when lizards first emerged in the morning and finished when the last lizard retired to its shelter in the evening. During observations, we recorded male and female identities in courtships and matings and male identities in agonistic encounters (win/lose; defined by when an individual retreated from another that was showing an agonistic display).

Additional information on the paternity analysis

For the 2010 enclosure experiment, DNA was isolated from tail-tip tissue using an ammonium acetate standard protocol (see Heathcote et al. 2015 for details), and for the 2013 experiment DNA was extracted with a DNeasy 96 Blood & Tissue Kit (Qiagen), following manufacturer's instructions (with overnight lysis). Given the limited number of potential fathers (eight per enclosure) we ran analyses using only nine microsatellites in 2010 and six microsatellite loci in 2013 (Table S2.3 for details). All loci were highly polymorphic with an average of 29.5 alleles per locus. Paternity was assigned using CERVUS v 3.0 (Marshall et al 1998). In each case, a simulation paternity analysis was performed based on 100,000 offspring and 8 candidate parents to estimate the critical values of the log-likelihood statistics (LOD scores). The proportion of typed loci was set to 99% in 2010 and 93% in 2013 based on allele frequency analysis with the proportion of loci mistyped as 0.04 in 2010 and 0.01 in 2013. The one parent known option was used, with all adult males in each enclosure population included as possible fathers and paternity was assigned based on the trio (mother, father, offspring) LOD score using a strict confidence level of 95%. In 2010, six females nested in the enclosures before removal and these clutches were retrieved and assigned full parentage using CERVUS. A total of 19 females, 8 of Western European origin and 11 of Italian origin, did not produce a clutch. Paternity of the 296 juveniles from 61 clutches was confirmed at >99% confidence. In 2013, a total of 14 females, 12 of Western European origin and 2 of Italian origin did not produce a clutch. As a consequence, there were a greater number of offspring from Italian females. Two embryos were excluded from the final paternity analysis because they were scored on fewer than 3 typed loci. From the remaining 201 offspring and embryos 18 mismatched at more than 1 locus with all fathers in their enclosure and were excluded from subsequent analyses.

Statistical Analysis

i) *Character divergence in allopatry:* We analysed differences between lineages in sexual dimorphism in these characteristics using linear mixed models in the lme4 package in R version 3.0.3 (R Development Core Team 2010). Each model included lineage, sex and their interaction as fixed factors and population of origin (nested within lineage) as a random effect with the exception of testes mass which did not include sex. For head length and testes mass we controlled for body size (snout-to-vent length) and for bite force we controlled for head length. Models for dorsal greenness and ventral blackness did not adhere to assumptions and were not suitable for transformation. For these traits we tested for sex and lineage differences separately using non-parametric Kruskal-Wallis tests.

ii) *Patterns of dominance, courtship and paternity upon secondary contact:* We ran separate models for the two enclosure experiments to test for differences in dominance, courtship frequency, overall reproductive success and the proportion of hybridization between the lineages. The dominance model was run using a linear mixed model in the lme4 package in R version 3.0.3 with male lineage and SVL as fixed factors. Because the dominance scores are not fully independent (as the dominance score of one individual depends on the dominance of the others in its enclosure) we assessed the robustness of these results using the permute package in R version 3.0.3, calculating P values by comparing the model coefficients against those of randomised datasets obtained through a Quadratic Assignment Procedure. Courtship number and overall reproductive success were run using generalized linear mixed models with a negative binomial distribution to correct for overdispersion in the glmmadmb package in R. Hybridization models were run using a generalized linear mixed model with a binomial distribution using the lmer package in R. All models included lineage and snout-to-vent length as fixed factors and enclosure as a random factor. Following the results for dominance, we also fitted the same models including dominance to separate lineage and dominance effects. We analyzed the phenotypic predictors of dominance by a running multiple regression model using

the lm function in R version 3.0.3. Head length, dorsal greenness and ventral blackness (standardized to a $\mu = 0$, $\sigma = 1$) were included as predictors. As several of these traits are highly correlated (Table S2.13) we confirmed our findings from the multivariate analysis by calculating univariate linear gradients (β_i) from linear mixed models (Lande and Arnold 1983). For the sperm competition trials we used a generalized linear mixed model with a binomial distribution and female lineage as a fixed factor and female identity as a random factor.

iii) *Fertility and viability of F1 hybrids:* To examine differences between crosses in the incidence of embryonic mortality we ran a generalized linear model with a binomial distribution and female lineage, male lineage and their interaction as fixed factors and clutch identity as a random effect. To examine differences between crosses in snout-to-vent length at hibernation and testes mass for males, we ran linear models with lineage cross (Western European, Italian, Hybrid), sex (for snout-to-vent length) and snout-to-vent length (for testes mass) as fixed factors. This analysis revealed that animals from the Western European lineage were smaller following hibernation (see results), which may explain why none of those females reproduced within the two months. Our test of hybrid fertility therefore contrasts hybrid females with F1 females of Italian origin, using a chi-square test for number of females producing eggs and a Kruskal-Wallis test for clutch size.

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Chapter 3

Experimental contact zones reveal the causes and targets of sexual selection in hybridizing lizards

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Author contributions: HEAM, TU and GMW conceived and designed the study. TU, GMW, HEAM, SM, PC, and GPL conducted field work. HEAM coordinated the enclosure experiment and collected behavioural data with JB. HEAM, GPL, and PC collected data in the lab. HEAM and SM generated microsatellite data. HEAM analysed the data and wrote the manuscript with input from all authors.

3.1 Abstract

Divergence in sexually selected traits in allopatry should affect the degree and direction of hybridization. However, few studies have established the causes and targets of sexual selection during secondary contact. Common wall lizards (*Podarcis muralis*) from north-central Italy have highly exaggerated male sexual traits compared to populations in Western Europe. Using experimental populations, we show that this creates asymmetries in male dominance, spatial habitat use, and reproductive success upon secondary contact. Hybridization occurred almost exclusively between males of the Italian lineage and females of the Western European lineage. We provide evidence to suggest stronger ongoing selection on male sexual traits within the dominant Italian lineage. However, these same characters did not predict hybridization, and hybrid matings contributed little to variance in male reproductive success. Instead, most hybrid offspring were sired by Italian males displaying phenotypes associated with lower within-lineage reproductive success. Thus, highly directional hybridization may arise in part because some Italian males are outcompeted within their own lineage but remain competitive relative to males of the other lineage. This pattern of hybridization is consistent with the direction of introgression in natural contact zones, but our data suggest that sexual selection acting through hybridization may be weak at the leading edge of natural hybrid zones.

Keywords: Behaviour, Hybridization, Introgression, Male-Male Competition, Podarcis

3.2 Introduction

Divergence in behavioural or morphological traits while populations are in allopatry can restrict gene flow between closely related lineages upon secondary contact (Coyne & Orr 2004). Genetic analyses of hybrid zones often, however, reveal directional patterns of introgression (e.g. Jezkova & Rodriguez-Robles 2013; Robbins *et al.* 2013). Sexual selection should be of particular importance within this context because the strength and direction of gene flow will depend upon mating behaviour and the propensity of individuals from each lineage to interact and hybridize in zones of secondary contact (e.g. Willis, Ryan & Rosenthal 2011; Charpentier *et al.* 2012). Pre-copulatory behaviours and morphology associated with mate acquisition and fertilization success can evolve rapidly under sexual selection and often show greater divergence among lineages than non-sexual characteristics (Panhuis et al. 2001; Mendelson & Shaw 2005). Consequently, the extent to which divergent sexual characteristics favour within- and between-lineage reproductive success can mediate patterns of hybridization. When one lineage has evolved advantageous sexual characteristics over the other, sexual selection may then act as the main driving force for genetic and phenotypic introgression (e.g. sexually selected introgression; Stein & Uy 2006).

Most existing studies of sexually selected introgression have implicated female choice as the main driver of gene flow (e.g. Stein & Uy 2006; Baldassarre & Webster 2013). However, male-male competition can overcome the effects of female mate preferences (e.g. Reichard *et al.* 2005), and cause extensive hybridization between taxa (Hartman *et al.* 2012; While *et al.* 2015a). Therefore, in species where males defend territories and compete for access to females, divergence in traits that influence the outcome of male-male competition could have consequences for which individuals are more likely to engage in hybridization, and determine the phenotypic targets of selection (e.g. Pearson & Rohwer 2000; Dijkstra & Groothuis 2011).

Despite this, surprisingly few studies have sought to quantify sexual selection upon secondary contact.

Previous work on the common wall lizard, *Podarcis muralis* (Laurenti 1768), has documented asymmetric gene flow between two lineages, across several regions of secondary contact (While *et al.* 2015a). The lineages, from the Italian Peninsula and from Western Europe, share a pattern of gene flow consistent with sexually selected introgression. Previous work also suggests that female choice based on male quantitative traits is absent or weak in this species (Heathcote *et al.* 2014), with no evidence that females of Western European or Italian origin discriminate between males of either lineage (Heathcote *et al.* 2016). This makes *P. muralis* a useful model for testing the role of sexual selection via male-male competition as a mediator of the strength and direction of hybridization. Here, we analyse data from experimental contact zones in outdoor enclosures to assess how phenotypic divergence between the lineages in male sexual traits causes asymmetric hybridization. We then assess the implications that this has for the strength and targets of sexual selection upon secondary contact.

3.3 Materials and Methods

Study animals

Common wall lizards, *Podarcis muralis,* are small (48-75 mm Snout-vent length (SVL)), diurnal, lacertids that are native to southern and central Europe. This species is strongly associated with human modified habitat (e.g. dry stone walls) and occupies a large geographic range (Schulte 2008; Salvi *et al.* 2013a; While *et al.* 2015a). Intraspecific diversity is high with several genetically and geographically distinct mitochondrial clades described (Giovannotti *et al.* 2010; Schulte *et al.* 2012a; Salvi *et al.* 2013a). The lineages that form the focus of this study represent two major mitochondrial clades that diverged approximately two million years ago (Gassert *et al.* 2013). Hereafter, animals referred to as from the Western European lineage fall within the Western France subclade and animals referred to as from the Italian lineage fall within the Tuscan haplotype clade (*sensu* Schulte *et al.* 2012a). The lineages differ in morphology (see below and Figure 3.1), and are often described as separate subspecies (Böhme 1986a). We captured 128 sexually mature lizards (> 48 mm SVL) in April 2013, from three localities in Tuscany, northern Italy (Prato (43°54'N, 11°06'E), Greve di Chianti (43°35'N, 11°19'E) and Colle di Val D'Elsa (43°25'N, 11°06'E)), and four localities in western France (Dinan (48°27'N, 2°02'W), Josselin (47°57'N, 2°32'W), Pontchateau (47°26'N, 2°05'W), Pouzagues (46°47'N, 0°50'E)). Upon capture, we sexed and toe-clipped each lizard for unique identification and measured four body size related morphological traits (SVL, Mass, Head Width and Head Length). We removed ~5 mm of tail tip tissue from every individual, which we preserved in 90% ethanol. Two authors (GMW and TU) gave each lizard a dorsal greenness score (Greenness) from 1 to 10 (1 being pure brown, 10 being pure green; correlation between observer scores = 0.98). One author (GMW) photographed all individuals on their ventral and lateral sides using a Canon EOS 350D digital camera. From the photographs, we quantified ventral blackness colouration (Blackness) and the area of the outer ventral scales (OVS) with blue colouration (OVS Blue Area).

Figure 3.1: Images of two male *Podarcis muralis* to show the typical Italian phenotype from north-central Italy (above) and Western European phenotype (below). Photos by Ben Halliwell and Guillem Pérez i de Lanuza**.**

We transported the lizards from the field in cloth bags (kept below 10 °C) to laboratory facilities at the Department of Zoology, University of Oxford, UK. There, we housed the lizards in plastic terraria (590 × 390 × 415 mm) under a 12:12 light/dark cycle, and provided them with six hours of UV lighting per day. Each terrarium contained a 60 W heat lamp, sand substrate, a brick basking site and shelter. Most females were fecund with their first clutch of the breeding season at time of capture (wall lizards lay up to three clutches per year). We kept the females that had not ovulated at capture (assessed using palpation, e.g. Gartrell *et al.* 2002) with a male during their receptive phase and all other lizards were kept individually. All females laid their first clutch in the lab prior to commencing the experiment.

Upon establishment in the laboratory, one author (GPL) objectively measured four chromatic traits from each male (OVS Hue, OVS UV Chroma, Dorsal Hue and Dorsal Green Chroma) using a USB-2000 portable Ocean Optics diode-array spectrometer and a PX-2 xenon strobe light source (Pérez i de Lanuza *et al.* 2014; see SI). We also measured maximum bite force (Bite Force) for all males and females, and mean testes mass for all males (Testes Mass), the latter of which was carried out at the completion of the experiment. See Supplementary Information for expanded details on the quantification of all morphological traits.

Experimental enclosures and behavioural data collection

We simulated the initial stage of secondary contact by releasing lizards into eight (~ 7 × 7 m) experimental enclosures at the John Krebs Field Station, University of Oxford. The climate in Oxford falls within the variation in the non-native range of wall lizards in England. We note that this study may be most representative of secondary contact zones in England, which are the result of introductions (Michaelides *et al.* 2015).

Within each enclosure, we created a gradient in habitat complexity by constructing three types of sites that varied in structural complexity and the opportunity for thermoregulation. Each site consisted of two stacked pallets (1.14 m^2) sandwiched with a sheet of felt underlay, but varied in the number and construction of concrete breezeblocks placed above the pallets, which acted as both a shelter and a thermal resource. We arranged high, medium and low quality pallets in a three by three organization from one side of the enclosure to the other (Figure S3.1).

At the start of the experiment we released 64 male lizards; four Italian (ITA) and four Western European (WEUR) males per enclosure. We monitored these males within their enclosures for at least nine days whilst they established territories (see below). We then released 64 females; four Italian and four Western European females per enclosure. With the exception of three females (added 1-3 days after), we released all female lizards into an enclosure simultaneously (see SI for further details on assignment to enclosures). Prior to release, we marked all lizards for identification at a distance with a unique number on their dorsal side using a non-toxic, non-hypoallergenic marker pen (Mitsubishi Pencil Company Ltd).

Two authors (HEAM and JB) monitored the eight enclosures during May and June 2013 to collect positional and interaction data (see SI & Table S3.2). This resulted in records of 5,638 positional and 1,138 social interaction observations. From the social interaction data, we classified 492 male-male interactions, 464 of which were deemed competitive, and 684 male-female interactions including 296 courtships and 65 matings. We retained competitive interactions, courtships and matings for analyses.

At the end of female gestation, we returned the lizards to laboratory facilities where females oviposited. We lost 15 female clutches from mortality (2 ITA, 2 WEUR), failure of the female to reproduce (10 WEUR), or failure to recapture (1 ITA) but were able to obtain reproductive output for two dead females via dissection. Western European females often produce only one

seasonal clutch in the native range so an absence of second clutch production by ten WEUR females was not surprising. For the remaining females, we retrieved and counted the number of eggs within each clutch, and noted the presence and number of infertile eggs (following Olsson & Shine 1997a). Two Italian females produced fully infertile clutches, and a further ten eggs from five females (3 ITA and 2 WEUR) were infertile or dumped but we included these when testing for differences in the potential reproductive output (i.e. clutch size) of Italian and Western European females.

We weighed each clutch and incubated fertile eggs at a constant 28 °C and humidity (5:1 vermiculite:water volume) until hatching. At hatching, we obtained tail tissue samples from all juveniles for paternity analysis, which were preserved in 90% ethanol. Average hatching success of offspring was 96% for Italian and 93% for Western European females, respectively. For ten of twelve aborted offspring we successfully extracted DNA and assigned paternity.

Paternity analysis

We isolated DNA from all adults and 203 offspring (hatchlings: 191, embryos: 12) using the DNeasy 96 Blood & Tissue Kit (Qiagen), following manufacturer's instructions (with overnight lysis). Given the limited number of potential fathers (eight per enclosure), we genotyped individuals at six microsatellite loci (Heathcote *et al.* 2015; Table S3). We assigned offspring paternity using Cervus 3.0 (Marshall *et al.* 1998; see SI). Twenty offspring (18 hatchlings and 2 embryos) could not be reliably assigned a father because they amplified at fewer than three loci or mismatched within their mother-father-offspring trio at more than one locus. This resulted in the retainment of 183 offspring for further analyses.

Statistical analyses

All statistical analyses unless otherwise stated were carried out in R 3.1.2 (Core Team 2014). We ran linear mixed models (LMMs) and generalized linear mixed models (GLMMs) for phenotypic, spatial, and behavioural analyses, including enclosure as a random effect when appropriate.

Spatial analyses

Spatial analyses were conducted in Ranges 8 (Kenward *et al.* 2008). We estimated home range areas from positional observations using a fixed-kernel contour analysis with a fixed smoothing parameter of 0.75 (Kie 2013; see SI). We calculated home range size, percentage overlap, and, for males, the number of overlapping females, at both the 50% (core home range) and 95% (total home range) isopleth level (Worton 1989; Table S4). We assigned each lizard to a pallet quality based on the location where the kernel estimate indicated peak density. We used the spread of the location distribution (the grand mean of distances between locations; Spencer & Barrett 1984) of each male's positional observations as an indicator of the extent to which males defend a territory (e.g. Morrison *et al.* 2002). We tested for lineage differences in home range area, male-female overlap, habitat quality, and spread. See SI for expanded details.

Social, behavioural and genetic network analyses

To determine whether social interactions and spatial distribution could mediate hybridization between the two lineages, we ran Mantel permutation analyses on behavioural, spatial and genetic association networks in the compiled version of SOCPROG 2.4 (Whitehead 2009). First, for each enclosure we tested for within-lineage assortativity in male-male competitive interactions, male-female courtships, observed matings, and paternity using social networks weighted by the total number of observed interactions (or for paternity, the number of offspring) between each dyad. Second, we tested for significant correlations between these

behavioural networks and core home range overlap (weighted by % overlap at the 50% isopleth) and paternity (weighted by numbers of offspring sired), respectively. All Mantel permutation analyses were based on 1,000 permutations, which achieved stability in p-values. For each set of analyses we combined the p-values for each enclosure into a single test statistic using Fisher's method (Fisher 1932).

Behavioural analyses

We calculated each male's dominance score (Dominance) based on David's method (David 1988), corrected for the numbers of interactions between dyads. We tested for significant differences between the lineages in Dominance with a LMM that included Lineage and SVL as fixed effects. The robustness of this result was confirmed through comparisons against randomised datasets, obtained via a Quadratic Assignment Procedure, based on 10,000 permutations of dominance scores per enclosure (Permute package, Simpson 2015).

Male reproductive success

We calculated the reproductive success of each male in terms of fertilization success (the total number of offspring sired) and mating success (the total number of female clutches including sired offspring) based on the paternity analysis. We excluded the use of behavioural observations of mating from the calculation of male mating success because of the potential for observation biases within and between the lineages. We examined male reproductive success separately by lineage because of differences between Italian and Western European females in the number of clutches produced, and the incidence of hybridization (see Results). Since the evolutionary consequences of selection will depend on relative rather than absolute reproductive success (Kingsolver & Pfennig 2007), we divided the fitness measures for each male by the mean for all males within his enclosure that were of the same lineage to generate relative measures of mating success and fertilization success for each male. The mean fitness

values within each enclosure were calculated with the inclusion of non-siring/unmated males (Shuster 2009).

Estimates of the strength and targets of sexual selection

We quantified the contribution of variance in relative within-lineage (W) and between-lineage (B) fertilization success to overall variance in male fertilization success following Webster et al. (1995). We use this as a proxy for their relative contribution to selection on male sexual traits. To quantify the relative strength of pre- and post-copulatory sexual selection, we further partitioned W and B into the (co)variance contributions of male mating success (M), mate fecundity (N) and paternity share (P) (Webster *et al.* 1995; see SI). In addition, we characterized the strength of pre-copulatory sexual selection on males with the Bateman gradient (β_{SS}), the slope of the least squares regression of relative mating success on relative fertilization success (Jones 2009). We compared Bateman gradients between lineages using a LMM with relative mating success, lineage, and their interaction as fixed effects, and enclosure as a random effect.

To identify the potential phenotypic targets for ongoing selection in Italian and Western European males, and to estimate the strength and direction of associations between traits and reproductive success, we performed multiple linear regression analyses with relative within-lineage fertilization success as the response variable, and standardized (within-lineage: mean = 0, SD =1) morphological traits and Dominance as fixed effects (Lande & Arnold 1983b). To quantify the associations between Italian male traits and hybridization, we performed the same analyses with relative between-lineage fertilization success as the response variable. The low incidence of hybridization involving Western European males precluded similar analyses (see Results). We collapsed SVL, Head Length, Head Width, and Body Mass into a single principle component (PC1_BodySize, Table S3.5). Furthermore, Dorsal Hue and Dorsal Green Chroma were replaced with the greenness score, which was highly correlated with both traits (Dorsal

Hue: r = - 0.88, Dorsal Green Chroma: r = 0.89). To avoid over parameterisation of our models, we performed the regression analyses on the nine remaining phenotypic traits separately for body size and performance related traits (Dominance, PC1_BodySize, Bite Force, Testes Mass) and colouration traits (Greenness, Blackness, OVS Blue Area, OVS Hue, OVS UV Chroma). We ran and evaluated all candidate models (Table S3.6, including single explanatory variables) based on the second order Akaike Information Criterion (AICc) and selected the top performing models as those < 2 ΔAICc from the best approximating model (Burnham & Anderson 2002). We report full/partial regression coefficients for traits in the top performing models and parameter estimates based on full-model averaging i.e. with shrinkage (Symonds & Moussalli 2011).

We supported our findings from multiple regression analyses by calculating standardized linear regression coefficients (β_i) from single-trait models controlling for SVL(Lande & Arnold 1983a). In addition, because associations between male phenotypic traits and reproductive success may be non-linear in form, we estimated standardized quadratic regression coefficients (γ_{ii}) as twice the coefficient for the second-order term from models including both linear and quadratic terms (Stinchcombe *et al.* 2008). We did not test for significant cross-product terms (i.e. correlational selection) to avoid over-fitting of the models relative to sample size.

3.4 Results

Morphological and spatial asymmetries between the lineages

Italian males had exaggerated phenotypes compared to Western European males, and several characters suggested to be under sexual selection in *Podarcis* lizards (e.g. Sacchi *et al.* 2009b; Huyghe *et al.* 2012) showed greater sexual dimorphism in the Italian lineage (Table 3.1, Table S3.1).

Table 3.1: Results from linear mixed models examining divergence and sexual dimorphism in body size, performance, and colouration between Italian and Western European lizards. A covariate; SVL, Head Length (HL), or Mass (M), was included in the models when appropriate, and population of origin nested within lineage was included as a random effect. Results for main effects are reported from models excluding non-significant interaction terms. Significant effects are highlighted in bold based on a threshold of $\alpha \leq 0.004$, adjusted from the nominal $\alpha < 0.05$ following Bonferroni correction for the number of tests performed on these data.

Response Variable	Lineage	Sex	Lineage × Sex	Covariate
SVL	$F_{1,4} = 0.00$, $p = 0.95$	$F_{1,123} = 5.28$, $p = 0.02$	$F_{1,122} = 5.21$, $p = 0.02$	
Head Length	**$F_{1,4} = 38.10$, $p < 0.002$**	**$F_{1,123} = 850.78$, $p < 0.001$**	$F_{1,121} = 1.85$, $p = 0.18$	**SVL: $F_{1,123} = 291.56$, $p < 0.001$**
Head Width	**$F_{1,4} = 101.63$, $p < 0.001$**	**$F_{1,123} = 291.63$, $p < 0.001$**	$F_{1,122} = 0.99$, $p = 0.32$	**SVL: $F_{1,121} = 187.08$, $p < 0.001$**
Mass	$F_{1,5} = 12.23$, $p = 0.02$	**$F_{1,123} = 43.69$, $p < 0.001$**	$F_{1,121} = 0.39$, $p = 0.54$	**SVL: $F_{1,123} = 227.95$, $p < 0.001$**
Bite Force	$F_{1,6} = 5.72$, $p = 0.04$	$F_{1,115} = 1.03$, $p = 0.31$	**$F_{1,115} = 10.02$, $p = 0.002$**	**HL: $F_{1,114} = 47.40$, $p < 0.001$**
Testes Mass	$F_{1,5} = 15.47$, $p = 0.01$			**M: $F_{1,56} = 12.74$, $p < 0.001$**
Dorsal Hue	**$F_{1,4} = 163.66$, $p < 0.001$**			SVL: $F_{1,48} = 0.06$, $p = 0.81$
Dorsal Green Chroma	**$F_{1,4} = 177.72$, $p < 0.001$**			SVL: $F_{1,48} = 1.57$, $p = 0.22$
Blackness	**$F_{1,5} = 25.80$, $p = 0.004$**	**$F_{1,121} = 47.44$, $p < 0.001$**	$F_{1,120} = 1.25$, $p = 0.27$	**SVL: $F_{1,121} = 10.21$, $p = 0.002$**
OVS Blue Area	**$F_{1,4} = 33.76$, $p = 0.004$**	**$F_{1,123} = 73.64$, $p < 0.001$**	$F_{1,121} = 0.77$, $p = 0.38$	SVL: $F_{1,123} = 4.44$, $p = 0.04$
OVS Hue	$F_{1,4} = 7.70$, $p = 0.05$			SVL: $F_{1,47} = 0.53$, $p = 0.47$
OVS UV Chroma	**$F_{1,4} = 67.59$, $p = 0.001$**			SVL: $F_{1,43} = 1.87$, $p = 0.18$

Males had larger core and total home ranges than females, but there were no significant differences between the lineages (Tables S3.4 and S3.8). Male core home ranges were not evenly distributed across habitat qualities and most males occupied either the high or low quality end of each enclosure (High (n=29), Medium (n=9), Low (n=25): $\chi^2 = 10.66$, $p = 0.004$), with the most dominant Italian males occupying high-quality sites (GLMM (Binomial) for Male Habitat Quality: Lineage: $\chi^2 = 6.95$, $p = 0.008$, Dominance: $\chi^2 = 0.18$, $p = 0.67$, Lineage × Dominance: $\chi^2 = 6.16$, $p = 0.01$). By contrast, female core home ranges were evenly distributed across habitat qualities (High (n=19), Medium (n=21), Low (n=24): $\chi^2 = 0.59$, $p = 0.74$). Consequently, there were no differences in male-females overlap between the lineages (Table S3.8). Italian males had similar clustering of observations regardless of the position of their core home range, whereas Western European males showed greater spread when the centre of their home range was a low-quality site (Origin: $F_{1,43} = 10.7$, $p < 0.001$, Habitat Quality: $F_{1,44} = 1.00$, $p = 0.32$: Origin × Habitat Quality: $F_{1,45} = 3.79$, $p = 0.06$).

Behavioural asymmetries between the lineages

Male-male competitive interactions were not assortative by lineage ($\chi^2 = 19.29$, $p = 0.24$, df = 16). Italian males were significantly more dominant than Western European males (ITA Males: 4.10 ± 0.13, WEUR Males: 2.81 ± 0.08, Lineage: $F_{1,53} = 60.87$, $p < 0.001$ SVL: $F_{1,56} = 5.84$, $p = 0.019$, (QAP: Lineage: $p < 0.001$, SVL: $p = 0.05$) and dominance was more strongly correlated with body size in the Italian lineage than in the Western European lineage (Table S3.9).

Italian males courted more females of both origins (ITA Females Courted: Lineage: $\chi^2 = 26.50$, $p < 0.001$, SVL: $\chi^2 = 0.39$, $p = 0.53$; WEUR Females Courted: Lineage: $\chi^2 = 20.22$, $p < 0.001$, SVL: $\chi^2 = 1.57$, $p = 0.21$) and, for Italian but not Western European males, Dominance was a strong predictor of both number of females courted (ITA Males: Dominance: $\chi^2 = 12.17$, $p < 0.001$, WEUR Males: Dominance: $\chi^2 = 0.47$, p=0.49) and number of courtships (ITA Males: Dominance: 93.69, $p < 0.001$, WEUR Males: Dominance: $\chi^2 = 0.51$, $p = 0.47$). Overall, courtship networks

were significantly assortative across enclosures (χ^2 = 28.28, p = 0.03, df = 16). Consequently, Italian females received more courtships on average than Western European females, and larger females received more courtships than smaller females (Female Lineage: χ^2 = 11.40, p < 0.001, Female SVL: χ^2 = 13.96, p <0.001). Sixty five of the 296 observed courtships resulted in an observed mating. In contrast to courtships, we found no assortativity by lineage in observed matings across enclosures (χ^2 = 9.0, p = 0.90, df = 16).

Reproductive success

There were no significant differences in clutch size between Italian and Western European females (Table 3.2, ITA: 4.81 ± 0.24, WEUR: 4.25 ± 0.32). Overall, the incidence of multiple paternity was higher for Western European females (ITA Clutches: 71% and WEUR Clutches: 85%), but there was no significant difference between the lineages in the number of fathers per clutch (Table 3.2, ITA Clutches: 2.04 ± 0.20, WEUR Clutches: 2.50 ± 0.18). Italian males sired significantly more offspring than Western European males (Lineage: χ^2 = 21.16, p < 0.001), but paternity was biased towards females of the same lineage (χ^2 = 60.04, p < 0.001, df = 16), and strongly predicted by both courtship networks (χ^2 = 69.57, p < 0.001, df = 16), and the percentage overlap in core home ranges between males and females (χ^2 = 35.27, p <0.001, df = 16). Thirty-four offspring were identified as hybrids, and the direction of hybridization was highly asymmetric (Table 3.2). Of the 104 offspring produced by ITA females 98 were sired by ITA males and 6 by WEUR males. Of the 79 offspring produced by WEUR females 51 were sired by WEUR males and 28 by ITA males.

Table 3.2: Results from generalized linear mixed models testing for lineage differences in clutch size, fathers per clutch, and proportion hybrid offspring. The effects of lineage on clutch size and fathers per clutch were tested with GLMMs (Poisson Error). The effect of lineage on proportion hybrid offspring was tested with a GLMM (Binomial Error). Female SVL was included as a covariate in all models, and clutch size as a covariate when analysing fathers per clutch. Significant effects are highlighted in bold.

Response Variable	Lineage	SVL	Clutch Size
Clutch Size	$\chi^2 = 1.60$, $p = 0.21$	$\chi^2 = 2.38$, $p = 0.12$	
Fathers per Clutch	$\chi^2 = 1.18$, $p = 0.28$	$\chi^2 = 0.64$, $p = 0.42$	$\chi^2 = 0.88$, $p = 0.35$
Proportion Hybrid Offspring	**$\chi^2 = 28.65$, $p < 0.001$**	$\chi^2 = 0.49$, $p = 0.48$	

Variance in male reproductive success and asymmetries in sexual selection

Within-lineage fertilization success was by far the greatest contributor to variance in male reproductive success with hybrid offspring responsible for less than 10% of the overall variance in male reproductive success in both lineages. For both lineages, variance in mating success (M) contributed most to overall variance in within-lineage fertilization success. Paternity share (P) made a 14% larger contribution to variance in success for Italian males than for Western European males. For both lineages, the contributions of mate fecundity (N) and the covariance among components were low (see Table S3.13 for variance contributions in full).

Males of both lineages had strong, positive Bateman gradients (ITA β_{SS}: 1.45, $CI_{95\%}$= 1.13, 1.77; WEUR β_{SS}: 1.02, $CI_{95\%}$= 0.79, 1.26) but the gradient was stronger for Italian males (Relative Mating Success: $F_{1,52} = 123.71$, $p < 0.001$, Lineage: $F_{1,52} = 3.48$, $p = 0.07$, Lineage × Relative Mating Success: $F_{1,52} = 5.03$, $p = 0.03$). For Italian males, within-lineage reproductive success was best explained by a model including Dominance and Testes Mass, and from the colouration traits, a model including OVS Hue and OVS UV Chroma (3. 3, see Tables S3.10, S3.11 and S3.12 for model selection tables in full). These conclusions were supported by single-trait models suggesting directional selection on Dominance, Testes Mass and OVS Hue, in addition to

disruptive selection on Greenness and OVS Blue Area (Table S3.14). For Western European males, five models with equal support suggested positive directional selection on body size and performance traits (Table 3.3; PC1_BodySize has negative factor loadings, Table S3.5). Only Ventral Blackness (positive coefficient) and OVS UV Chroma (negative coefficient) were retained from multiple regression analyses on Western European colouration traits, but the null model was equally well supported (Table 3.3). The conclusion that directional selection on the colouration of Western European males is weak was supported by single-trait models (Table S3.14).

Table 3.3: Top supported models (< 2 ΔAICc) from multiple regression analyses to assess the best phenotypic predictors of relative within-lineage fertilization success of Italian and Western European males. For each model, the number of parameters (K), the relative likelihood (AICcWt), and the evidence ratio with reference to the best approximating model (ER), are reported. When not included within the top supported models, AICc values for the Null model are presented in italics for comparison. Regression coefficients (β) and bootstrap estimates of 95% confidence intervals ($CI_{95\%}$) are reported for all traits in the models. Model averaged parameter estimates (model-averaged β) and unconditional 95% confidence intervals (unconditional $CI_{95\%}$) are also presented, generated via full-model averaging based on all candidate models.

Lineage	Analyses	Model	K	AICc	ΔAICc	AICcWt	ER	Trait	β	$CI_{95\%}$	Model-averaged β	Unconditional $CI_{95\%}$
ITA	Body Size and Performance (n = 29)	8	5	89.09	0	0.26	1.00	Dominance	0.33	-0.04 0.68	0.23	-0.25 0.72
								Testes Mass	0.42	0.06 0.78	0.39	-0.11 0.89
		5	4	89.30	0.21	0.24	1.11	Testes Mass	0.53	0.17 0.89		
		1	*3*	*94.09*	*5.00*	*0.02*	*12.18*	*Null*				
	Colouration (n = 30)	16	5	92.82	0	0.33	1.00	OVS Hue	-0.63	-1.03 -0.24	-0.48	-1.07 0.11
								OVS UV Chroma	-0.48	-0.87 -0.08	-0.31	-0.86 0.25
		1	*3*	*96.36*	*3.54*	*0.06*	*5.87*	*Null*				
WEUR	Body Size and Performance (n = 30)	5	4	89.46	0	0.22	1.00	Testes Mass	0.26	-0.06 0.60	0.13	-0.22 0.48
		2	4	90.60	1.14	0.12	1.77	Dominance	0.17	-0.15 0.54	0.08	-0.21 0.36
		3	4	90.74	1.28	0.11	1.90	PC1_BodySize	-0.18	-0.52 0.17	-0.05	-0.29 0.20
		8	5	90.80	1.34	0.11	1.95	Dominance	0.21	-0.12 0.55		
								Testes Mass	0.28	-0.06 0.61		
		4	4	90.94	1.48	0.10	2.10	Bite Force	0.16	-0.18 0.51	0.03	-0.19 0.25
		1	*3*	*94.31*	*4.85*		11.33	*Null*				
	Colouration (n = 24)	6	4	72.57	0	0.17	1.00	OVS UV Chroma	-0.33	-0.72 0.02	-0.17	-0.59 0.25
		1	3	72.75	0.18	0.16	1.09	Null				
		13	5	73.35	0.78	0.12	1.48	Blackness	0.28	-0.06 0.63	0.12	-0.25 0.49
								OVS UV Chroma	-0.33	-0.68 0.02		
		3	4	73.43	0.85	0.11	1.53	Blackness	0.29	-0.09 0.66		

For between-lineage fertilization success of Italian males, the best supported models for colouration suggested the opposite direction of associations with OVS UV Hue (positive coefficient) and Greenness (negative coefficient); however, the null model was equally well supported (Table 3.4). Similarly, single-trait models for between-lineage fertilization success indicated a general trend for reversed direction when compared to within-lineage fertilization success (Figure 3.2, Table S3.14).

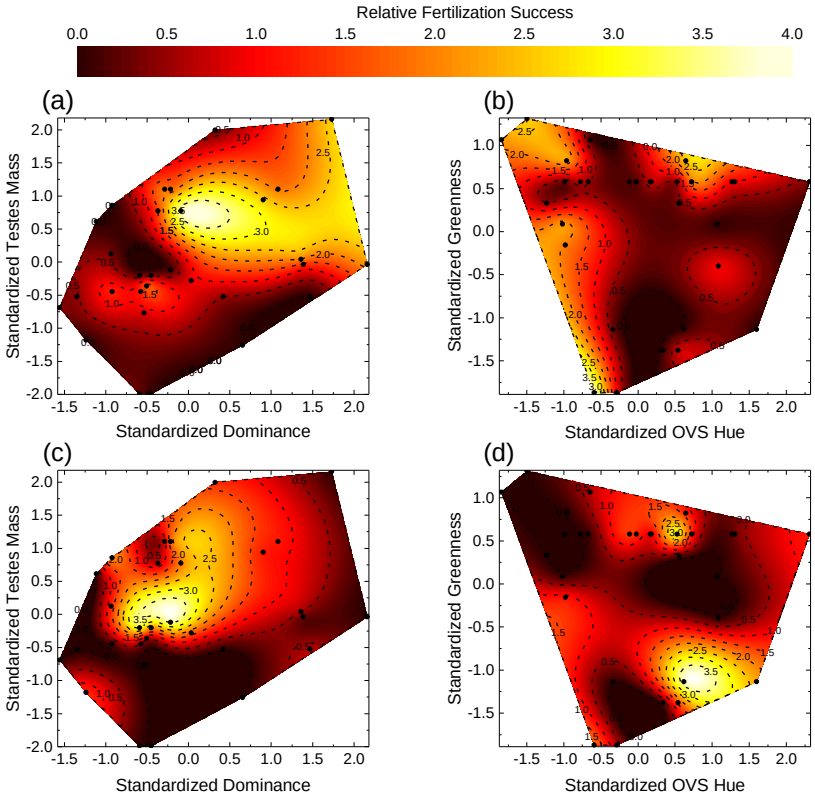

Figure 3.2: Contour plots for Italian males depicting the relationships between four sexually selected traits (Dominance and Testes Mass, shown in (a) and (c), and Greenness and OVS UV Hue, shown in (b) and (d)) and relative within-lineage ((a) and (b)) or between- lineage ((c) and (d)) fertilization success (colour gradient: dark red = 0, light yellow = 4). Plots are shown to illustrate differences in the form and direction of associations when comparing within-lineage and between-lineage fertilization success. Contours were predicted by triangulation of the data points followed by linear interpolation.

Table 3.4: Top supported models (< 2 ΔAICc) from multiple regression analyses to assess the best phenotypic predictors of relative between-lineage fertilization success of Italian males. For each model, the number of parameters (K), the relative likelihood (AICcWt), and the evidence ratio with reference to the best approximating model (ER), are reported. Regression coefficients (β) and bootstrap estimates of 95% confidence intervals ($CI_{95\%}$) are reported for all traits in the models. Model-averaged parameter estimates (model-averaged β) and unconditional 95% confidence intervals (unconditional $CI_{95\%}$) are also presented, generated via full-model averaging based on all candidate models.

Lineage	Analyses	Model	K	AICc	ΔAICc	AICcWt	ER	Trait	β	$CI_{95\%}$	Model Averaged β	Unconditional $CI_{95\%}$
ITA	Body Size and Performance (n = 29)	1	3	95.07	0	0.29	1.00	Null				
		5	4	96.74	1.67	0.13	1.88	Testes Mass	0.21	-0.18 0.61	0.14	-0.27 0.54
	Colouration (n = 30)	5	4	97.16	0	0.13	1.00	OVS Hue	0.35	-0.04 0.73	0.12	-0.27 0.51
		1	3	97.63	0.47	0.10	1.26	Null				
		10	5	97.75	0.59	0.09	1.35	Greenness	-0.36	-0.74 0.01	-0.16	-0.60 0.27
								OVS UV Chroma	-0.33	-0.71 0.05	-0.09	-0.44 0.26
		2	4	97.77	0.61	0.09	1.35	Greenness	-0.32	-0.70 0.08		
		9	5	98.24	1.08	0.07	1.71	Greenness	-0.26	-0.64 0.12		
								OVS Hue	0.30	-0.07 0.68		
		6	4	98.37	1.20	0.07	1.83	OVS UV Chroma	-0.28	-0.67 0.12		

3.5 Discussion

Phenotypic divergence is typically expected to reduce the likelihood of hybridization between taxa in sympatry (Coyne & Orr 2004). However, this may not apply in contact zones between lineages that are in intermediate stages of divergence (Coyne & Orr 1989). In fact, rather than limit gene exchange, phenotypic differences in sexually selected traits may actively promote hybridization in a given direction (e.g. Parsons *et al.* 1993b; Baldassarre & Webster 2013). Experimental demonstration of highly asymmetric hybridization between lineages of the common wall lizard, *Podarcis muralis,* is consistent with historical differences in the strength of sexual selection, which makes males of one lineage competitively superior (While et al. 2015). However, our results reveal that most hybrid offspring were sired by males of the dominant lineage displaying traits associated with relatively low reproductive success with females of their own lineage. The results are consistent with the direction of introgression in regions of secondary contact (While *et al.* 2015a), but, together with the finding that hybridization contributed little to variance in fertilization success, suggest that the strength of sexual selection operating through hybridization may be relatively weak at the leading edge of natural hybrid zones.

As predicted given the differences in male morphology and behaviour, Italian males were strongly dominant over Western European males and achieved greater reproductive success (over and above differences via fewer second clutches from Western European females). In addition, the within-lineage Bateman gradient was steeper, and selection on sexual traits was stronger, for Italian males. Consistent with the more pronounced sexual dimorphism in the Italian lineage, dominance and body colouration strongly predicted reproductive success for Italian but not for Western European males. Sexual dichromatism is positively correlated with sexual size dimorphism across a wide range of lacertid lizards, and is probably driven by intrasexual selection (Pérez i de Lanuza *et al.* 2013). Our data support that UV colouration may

act as sexual ornamentation in wall lizards. Moreover, our results suggest that there is likely to be stronger contemporary sexual selection on colouration traits in populations of the Italian lineage compared to the Western European lineage. However, some spectral variables of UV-blue outer ventral scales are also positively correlated with fighting ability and body condition in Western European males (Pérez i de Lanuza *et al.* 2014). This implies that Western European males will respond to the same colour signals as Italian males, which gives Italian males an advantage in male-male competition. The higher reproductive success for males with larger testes may also point towards a competitive advantage for Italian males in sperm competition (Birkhead & Møller 1998).

In response to this competitive social environment, our space use and behavioural data suggests that some Western European males modify their behaviour and adopt a 'floater' strategy (Oliveira *et al.* 2008). In contrast, the least dominant Western European males were apparently tolerated within the territories of the most dominant Italian males. Conditional behavioural tactics have been demonstrated in lizards (e.g. Noble *et al.* 2013). However, in our system, neither strategy appears to allow Western European males access to females of the opposite lineage, creating close to unidirectional hybridization (sensu Wirtz 1999b). These results could predict that exaggerated sexually selected male traits will increase reproductive success with females of the opposite lineage. However, we show that selection on male quantitative traits through hybridization was weak, or even reversed in sign. This pattern could arise if females of the Western European lineage preferred subdominant Italian males (see Rosenthal 2013). Although we cannot completely exclude this explanation, previous work has shown that female discrimination of males with different quantitative characters is weak or absent (Heathcote et al. 2014), to the extent that females do not even discriminate between males of the two lineages (Heathcote et al. 2016). Therefore, these patterns of hybridization are more likely to be driven by males. Since courtships indicate that males prefer females of their own lineage, subdominant Italian males should be excluded from access to preferred, Italian, females and, therefore, more

prone to hybridize. Asymmetric patterns of hybridization could then arise because the less dominant Italian males are still competitively superior over Western European males; which is supported by the dominance hierarchies in the enclosures. More generally, when males of different lineages recognize each other as competitors, as in wall lizards, asymmetric hybridization should be especially pronounced if one lineage has a consistent competitive advantage over the other (e.g. Pearson & Rohwer 2000; Rosenfield & Kodric-Brown 2003).

Combined, our results suggest that individuals with exaggerated sexual traits may not promote directional introgression at the leading edge of the hybrid zone. Despite this, previous work has documented directional nuclear gene flow from the Italian lineage into the Western European lineage and even greater introgression of sexual traits (head size, and dorsal and ventral colouration (While *et al.* 2015a). There are several explanations that could account for the observed patterns of introgression despite our experimental findings. Firstly, males with Italian phenotypes should have high rates of hybridization if the population is biased towards Western European individuals since encounter rates with preferred Italian females will be low. Hybridization involving dominant males could be further enhanced by a reduction in Italian male mate preferences in response to low encounters with Italian females (e.g. Willis *et al.* 2011; Verzijden *et al.* 2012). Indeed, in non-native hybrid zones in Germany, the introduction of small numbers of Italian lizards has resulted in extensive introgression into native Western European populations (Schulte *et al.* 2012b; While *et al.* 2015a). Furthermore, results from theoretical models (e.g. Currat & Excoffier 2005) suggest that introgression could be extensive, even when hybridization is limited, due to competitive displacement or in an expanding hybrid zone (Buggs 2007). Secondly, as the female population becomes more admixed, male mate preferences for their own lineage will no longer limit the overall reproductive success of males with exaggerated sexual traits. In addition, since subdominant males may be young rather than intrinsically low quality, the quantitative measures of their sexual characters at the time of hybridization may not represent their true breeding values for those traits (Pemberton 2010).

Thus, hybrid male offspring could exhibit highly exaggerated sexual characters and be highly competitive even if sired by subdominant fathers. These potential influences make it difficult to predict how variation in the propensity to hybridize among Italian males will influence the broader geographic patterns of introgression in native and non-native regions of secondary contact. Detailed studies of phenotypic variation and selection at the leading edge of natural hybrid zones would be interesting in this regard.

In summary, our results highlight how behavioural interactions among individuals can shape hybridization. We demonstrated experimentally that asymmetries in male-male competitive ability are sufficient to promote asymmetric hybridization between lineages of wall lizards upon secondary contact, an initial step towards asymmetric gene flow. However, sexual selection on male traits through hybridization is likely to be weak at the leading edge of the hybrid zone. If, and how, this will influence the introgression of genetic and phenotypic characters requires further study.

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3.8 Supplementary Figures

Figure S3.1: Photograph of an enclosure used to house lizards during the experiment.

3.9 Supplementary Tables

Table S3.1: Mean trait values presented by lineage and sex for thirteen morphological traits. Values are based on measurements taken from the 128 lizards in this study.

		Males				Females			
	Trait	ITA		WEUR		ITA		WEUR	
		Mean	± 1 SE	Mean	± 1 SE	Mean	± 1 SE	Mean	± 1 SE
Body Size and Performance	SVL (mm)	62	1	60	1	59	1	60	1
	Head Width (mm)	7.9	0.7	7.1	0.5	6.6	0.1	6.1	0.1
	Head Length (mm)	16.3	0.2	15.0	0.2	13.1	0.1	12.6	0.1
	Mass (g)	6.24	0.24	5.24	0.18	4.84	0.18	4.38	0.17
	Bite Force (N)	7.66	0.53	4.41	0.32	2.92	0.13	2.60	0.09
	Testes Mass (g)	0.031	0.001	0.020	0.002				
Colouration	Greenness Score (1-10)	7	0.4	1	0.1	6	0.5	1	0
	Dorsal Green Chroma	0.27	0.01	0.19	0.00				
	Dorsal Hue (nm)	584	3.80	651	3.16				
	Ventral Blackness (%)	45.3	1.86	15.9	1.87	11.7	2.07	7.1	1.25
	OVS Blue Area (mm^2)	10.5	1.1	5.5	0.7	3.8	0.5	0.9	0.2
	OVS UV Chroma	0.38	0.03	0.31	0.02				
	OVS Hue (nm)	364	1.22	369	0.79				

Table S3.2: Ethogram used during behavioural observations to collect data on male-male and male-female interactions.

Behaviour	Description	Code
Basking	Basking	1
Approach	Walking directly towards another individual at a slow pace	2
Charge	Sudden, short, and fast paced approach directly towards another individual (aggressive)	3
Display	(1) Male-male aggression: (a) throat puffed out, (b) exposure of outer ventral scales on lateral flanks directly towards another male either stationary or with a sideways approach, (c) forehead pointing down towards substrate and shoulders raised on approach to a male. (2) Courtship: forehead pointing towards substrate and shoulders raised on approach to a female	4
Alert	Stationary with front legs extended, and head and forebody raised	5
Attack	Successful or unsuccessful attempt to bite another individual (excluding tail grab)	6
Head Grasp	One male with jaws locked around the upper jaw of another male (rare escalation of violence, typically following an extended period of displaying by two males of similar body size)	7
Chase/Follow	Extended pursuit of another individual	8
Retreat	(1) Submissive movement away from the vicinity of another individual (usually under cover) preceded by looking directly towards that individual. (2) Escape undercover following aggressive behaviour by another individual	9
Freeze	Abrupt halt to previous behaviour followed by no movement (typically leads to a retreat)	10
Wave	In sight of another lizard, rapid movement of front leg(s) either onto the substrate or in the air (aggressive or submissive)	11
Tail Quiver	Female shaking her tail rapidly in response to the presence of a male or in response to a tail grab	12
Tail Grab	A male attaching his jaws onto the tail of a female prior to copulation	13
Male tongue flick on female	Male flicking his tongue towards a female	14
Mating	Two lizards copulating	15
Female back pat	Female lies by male with one front leg on his back	16
Defecate	Defecate	17
Moving/Patrolling	Moving/Patrolling	18
Male alert by Female	Alert by female	19
Fight	Aggression between individuals with physical contact	20
Hunting/Feeding	Observations of feeding behaviours	21
Male-Female Lying Together	Male and female lying side by side	22

Table S3.3: Details on the six microsatellites used for the analysis of offspring paternity. Primers were combined within two multiplexes. Numbers represent base pairs (bp).

Multiplex*	Locus		Primer sequence (5'-3')	Product size (bp)	Repeat motif	Range (bp)
1	PmurC150	F	[6-FAM]GTCAGCTTTGCAGCACCTTAG	193	CA	171-217 [odd]
		R	GCGATTAGAGAAGGCGTTTG			
	PmurC168	F	[HEX]GGTCCGGCTTCAAAGAATAAG	244	TTTC	210-306 [even]
		R	CAGAGGACTCGCTCAAGGAC			
	PmurC275_278	F	[6-FAM]GCTTAAAATTAATGCTGCTATTGTATC	245	TATC	219-610 [odd]
		R	ATAGGTAGAAAATTTATAAACCCTTGG			
2	PmurC164	F	[6-FAM]ATCGATGAATGAAGGGCAGT	216	GATA	170-246 [even]
		R	CCAGGCATTGTCAAACTATCTG			
	PmurC038	F	[HEX]CAATGTGCAGTGTTGGGTTG	210	TATC	193-425 [odd]
		R	ATGTGAGCGACTCCTGGATG			
	PmurC028	F	[6-FAM]TTGCTTCTGAATACGCCTAGC	287	TATC	253-543 [odd]

*multiplexes developed by Heathcote et al. (2015)

Table S3.4: Mean (± 1 SE) core home range area (50% Isopleth Area) and total home range area (95% Isopleth Area) for lizards during the enclosure experiment. Values are presented by lineage and sex. Mean number of positional observations recorded during the experiment are also reported for each category.

Lineage	Sex	Core Home Range (m^2)	Total Home Range (m^2)	Observations
ITA	Male	4.2 ± 0.3	17.7 ± 1.1	78 ± 4
	Female	2.2 ± 0.2	9.5 ± 1.1	34 ± 3
WEUR	Male	4.7 ± 0.3	19.5 ± 1.2	44 ± 3
	Female	2.5 ± 0.3	9.5 ± 1.1	26 ± 2

Table S3.5: Factor loadings and the proportion of variance explained by the first principal component (PC1_BodySize) from a principle component analysis on strongly correlated measures of male body size (SVL, Body Mass, Head Length and Head Width). Analyses were performed separately by lineages. PC1_BodySize was included in multiple regression analyses to assess the best phenotypic predictors of male reproductive success.

Lineage	Proportion Variance	PC1_BodySize Factor Loadings			
		SVL	Mass	Head Length	Head Width
ITA	0.95	-0.92	-0.27	-0.26	-0.12
WEUR	0.97	-0.95	-0.22	-0.19	-0.11

Table S3.6: Candidate models from multiple regression analyses to identify phenotypic traits associated with relative within-lineage and relative between-lineage male fertilization success. All models included Enclosure as a random effect.

Analyses	Candidate Model	Standardized Predictors
Body Size and Performance	1	Null
	2	Dominance
	3	PC1_BodySize
	4	Bite Force
	5	Testes Mass
	6	Dominance + PC1_BodySize
	7	Dominance + Bite Force
	8	Dominance + Testes Mass
	9	PC1_BodySize + Bite Force
	10	PC1_BodySize + Testes Mass
	11	Bite Force + Testes Mass
	12	Dominance + PC1_BodySize + Bite Force
	13	Dominance + PC1_BodySize +Testes Mass
	14	Dominance + Bite Force + Testes Mass
	15	PC1_BodySize + Bite Force + Testes Mass
	16	Dominance + PC1_BodySize + Bite Force + Testes Mass
Colouration	1	Null
	2	Greenness
	3	Blackness
	4	OVS Blue Area
	5	OVS Hue
	6	OVS UV Chroma
	7	Greenness + Blackness
	8	Greenness + OVS Blue Area
	9	Greenness + OVS Hue
	10	Greenness + OVS UV Chroma
	11	Blackness + OVS Blue Area
	12	Blackness + OVS Hue
	13	Blackness + OVS UV Chroma
	14	OVS Blue Area + OVS Hue
	15	OVS Blue Area + OVS UV Chroma
	16	OVS Hue + OVS UV Chroma
	17	Greenness + Blackness + OVS Blue Area
	18	Greenness + Blackness + OVS Hue
	19	Greenness + Blackness + OVS UV Chroma
	20	Greenness + OVS Blue Area + OVS Hue
	21	Greenness + OVS Blue Area + OVS UV Chroma
	22	Greenness + OVS Hue + OVS UV Chroma
	23	Blackness + OVS Blue Area + OVS Hue
	24	Blackness + OVS Blue Area + OVS UV Chroma
	25	Blackness + OVS Hue + OVS UV Chroma
	26	OVS Blue Area + OVS Hue + OVS UV Chroma
	27	Greenness + Blackness + OVS Blue Area + OVS Hue
	28	Greenness + Blackness + OVS Blue Area + OVS UV Chroma
	29	Greenness + Blackness + OVS Hue + OVS UV Chroma
	30	Greenness + OVS Blue Area + OVS Hue + OVS UV Chroma
	31	Blackness + OVS Blue Area + OVS Hue + OVS UV Chroma
	32	Greenness + Blackness + OVS Blue Area + OVS Hue + OVS UV Chroma

Table S3.7: Variance Inflation Factors (VIFs) for traits in multiple regression analyses to identify phenotypic traits associated male fertilization success. VIFs were < 2 in all cases.

	Body Size and Performance Traits				Colouration Traits				
	Dominance	PC1_BodySize	Bite Force	Testes Mass	Greenness	Blackness	OVS Blue Area	OVS Hue	OVS UV Chroma
ITA	1.57	1.86	1.64	1.31	1.37	1.24	1.15	1.73	1.53
WEUR	1.17	1.38	1.55	1.24	1.67	1.40	1.33	1.42	1.20

Table S3.8: Results from tests for asymmetries between the lineages in home range area and male-female overlap. Results for main effects are reported from models excluding non-significant interaction terms. All models included Enclosure as a random effect. Significant effects are in bold.

Response Variable	Lineage	Sex	Lineage × Sex
Core Home Range Area	$F_{1,117} = 1.81$, p = 0.18	**$F_{1,117} = 77.35$, p < 0.001**	$F_{1,116} = 0.07$, p = 0.79
Total Home Range Area	$F_{1,117} = 0.62$, p = 0.43	**$F_{1,117} = 91.17$, p < 0.001**	$F_{1,116} = 0.69$, p = 0.41
Observations*	**$F_{1,123} = 59.94$, p < 0.001**	$F_{1,13} = 0.39$, p = 0.54	**$F_{1,113} = 13.16$, p < 0.001**
	Lineage	Dominance	Lineage × Dominance
Overlapping Females	$\chi^2 = 0.45$, p = 0.50	$\chi^2 = 0.79$, p = 0.37	$\chi^2 = 0.22$, p = 0.64
Overlapping Same Lineage Females	$\chi^2 = 1.15$, p = 0.28	$\chi^2 = 0.48$, p = 0.49	$\chi^2 = 1.65$, p = 0.20
Overlapping Other Lineage Females	$\chi^2 = 0.03$, p = 0.87	$\chi^2 = 0.89$, p = 0.34	$\chi^2 = 0.04$, p = 0.84

* For analysis of number of observations, we controlled for number of observation periods (**$F_{1,11} = 5.82$, p = 0.04**)

Table S3.9: Pearson's correlation coefficients between male traits including dominance. Upper triangle is for Italian males and lower triangle is for Western European males.

	Dominance	SVL	Body Mass	Head Length	Head Width	Bite Force	Testes Mass	Dorsal Hue	Dorsal Green Chroma	Blackness	OVS Blue Area	OVS Hue	OVS UV Chroma
Dominance		0.56	0.60	0.56	0.57	0.48	0.32	-0.06	0.22	-0.05	0.18	-0.33	0.30
SVL	-0.11		0.84	0.91	0.81	0.54	0.42	-0.05	0.22	0.10	0.29	0.06	-0.17
Body Mass	-0.04	0.76		0.87	0.75	0.63	0.51	0.04	0.09	0.12	0.43	0.16	-0.27
Head Length	0.03	0.79	0.71		0.78	0.62	0.37	-0.16	0.30	0.09	0.50	0.10	-0.12
Head Width	-0.01	0.86	0.75	0.79		0.66	0.48	0.02	0.13	0.12	0.33	0.13	-0.18
Bite Force	0.38	0.39	0.25	0.53	0.37		0.43	-0.22	0.30	-0.07	0.52	-0.09	-0.21
Testes Mass	0.03	0.24	0.31	0.38	0.20	0.21		0.11	-0.04	0.08	0.18	-0.06	-0.22
Dorsal Hue	0.11	-0.37	0.11	-0.32	-0.33	-0.55	-0.05		-0.92	-0.11	-0.23	0.42	-0.05
Dorsal Green Chroma	-0.21	0.36	0.02	0.26	0.35	0.27	0.03	-0.62		0.03	0.24	-0.49	0.09
Blackness	0.21	0.53	0.55	0.47	0.48	0.48	0.19	-0.25	0.14		0.08	0.31	-0.15
OVS Blue Area	-0.01	-0.16	-0.18	0.05	-0.05	-0.29	0.11	0.11	-0.09	-0.44		0.11	-0.26
OVS Hue	-0.24	0.35	0.04	0.18	0.08	0.02	0.29	-0.04	0.12	0.07	0.01		-0.50
OVS UV Chroma	0.49	0.11	0.09	0.13	0.08	0.32	0.17	-0.14	-0.04	-0.06	0.22	-0.06	

Table S3.10: Complete model selection tables from multiple regression analyses to assess the best phenotypic predictors of relative within-lineage fertilization success for Italian males. The top supported models (< 2 ΔAICc) are highlighted.

	Italian Males Relative Within- Lineage Fertilization Success						
	Candidate Model	K	AICc	Delta AICc	AICc Weight	Cumulative Wt	Log Likelihood
Body Size and Performance	8	5	89.09	0	0.26	0.26	-38.24
	5	4	89.3	0.21	0.24	0.5	-39.82
	2	4	91.14	2.05	0.1	0.6	-40.74
	13	6	91.31	2.21	0.09	0.69	-37.74
	14	6	91.47	2.38	0.08	0.77	-37.83
	11	5	92.23	3.14	0.06	0.82	-39.81
	10	5	92.24	3.14	0.05	0.88	-39.81
	6	5	94.05	4.96	0.02	0.9	-40.72
	7	5	94.08	4.98	0.02	0.92	-40.73
	1	3	94.09	5	0.02	0.94	-43.57
	16	7	94.43	5.34	0.02	0.96	-37.55
	3	4	95.41	6.32	0.01	0.97	-42.87
	15	6	95.42	6.33	0.01	0.98	-39.8
	4	4	95.73	6.63	0.01	0.99	-43.03
	12	6	97.26	8.17	0	1	-40.72
	9	5	98.14	9.05	0	1	-42.77
Colouration	16	5	92.82	0	0.33	0.33	-40.16
	5	4	95.13	2.31	0.1	0.43	-42.77
	22	6	95.33	2.51	0.09	0.52	-39.84
	26	6	95.7	2.88	0.08	0.6	-40.02
	25	6	95.97	3.15	0.07	0.67	-40.16
	1	3	96.36	3.54	0.06	0.72	-44.72
	14	5	97.12	4.3	0.04	0.76	-42.31
	9	5	98.02	5.2	0.02	0.79	-42.76
	12	5	98.02	5.21	0.02	0.81	-42.76
	30	7	98.14	5.32	0.02	0.83	-39.52
	6	4	98.45	5.63	0.02	0.85	-44.43
	4	4	98.49	5.67	0.02	0.87	-44.44
	3	4	98.63	5.81	0.02	0.89	-44.51
	29	7	98.72	5.9	0.02	0.91	-39.82
	2	4	98.97	6.15	0.02	0.92	-44.68
	31	7	99.13	6.31	0.01	0.94	-40.02
	20	6	100.07	7.26	0.01	0.95	-42.21
	23	6	100.25	7.43	0.01	0.95	-42.3
	13	5	100.76	7.94	0.01	0.96	-44.13
	11	5	100.88	8.06	0.01	0.97	-44.19
	15	5	101.01	8.19	0.01	0.97	-44.25
	18	6	101.17	8.35	0.01	0.98	-42.76
	10	5	101.33	8.51	0	0.98	-44.41
	7	5	101.34	8.52	0	0.98	-44.42
	8	5	101.38	8.57	0	0.99	-44.44
	32	8	101.84	9.03	0	0.99	-39.49
	24	6	103.51	10.69	0	0.99	-43.93
	27	7	103.51	10.7	0	1	-42.21
	19	6	103.79	10.97	0	1	-44.07
	17	6	103.99	11.17	0	1	-44.17
	21	6	104.16	11.34	0	1	-44.25
	28	7	106.92	14.1	0	1	-43.91

Table S3.11: Complete model selection tables from multiple regression analyses to assess the best phenotypic predictors of relative within-lineage fertilization success for Western European males. The top supported models (< 2 ΔAICc) are highlighted.

	Western European Males Relative Within- Lineage Fertilization Success						
	Candidate Model	K	AICc	Delta AICc	AICc Weight	Cumulative Wt	Log Likelihood
Body Size and Performance	5	4	89.46	0	0.22	0.22	-39.93
	2	4	90.6	1.14	0.12	0.34	-40.5
	3	4	90.74	1.28	0.11	0.45	-40.57
	8	5	90.8	1.34	0.11	0.57	-39.15
	4	4	90.94	1.48	0.1	0.67	-40.67
	10	5	92.07	2.62	0.06	0.73	-39.79
	11	5	92.19	2.73	0.06	0.78	-39.84
	6	5	92.27	2.82	0.05	0.84	-39.89
	7	5	93.08	3.62	0.04	0.87	-40.29
	9	5	93.37	3.91	0.03	0.9	-40.44
	13	6	93.6	4.14	0.03	0.93	-38.97
	14	6	93.95	4.49	0.02	0.95	-39.15
	1	3	94.31	4.85	0.02	0.97	-43.73
	15	6	95.17	5.71	0.01	0.98	-39.76
	12	6	95.41	5.95	0.01	0.99	-39.88
	16	7	96.97	7.51	0.01	1	-38.94
Colouration	6	4	72.57	0	0.17	0.17	-31.23
	1	3	72.75	0.18	0.16	0.33	-32.78
	13	5	73.35	0.78	0.12	0.44	-30.01
	3	4	73.43	0.85	0.11	0.56	-31.66
	4	4	75.52	2.94	0.04	0.6	-32.71
	16	5	75.52	2.94	0.04	0.64	-31.09
	5	4	75.54	2.97	0.04	0.67	-32.72
	2	4	75.63	3.05	0.04	0.71	-32.76
	10	5	75.73	3.16	0.04	0.75	-31.2
	15	5	75.8	3.23	0.03	0.78	-31.23
	7	5	76.24	3.66	0.03	0.81	-31.45
	24	6	76.31	3.74	0.03	0.84	-29.69
	12	5	76.47	3.89	0.02	0.86	-31.57
	25	6	76.55	3.98	0.02	0.88	-29.8
	11	5	76.56	3.98	0.02	0.91	-31.61
	19	6	76.92	4.34	0.02	0.93	-29.99
	14	5	78.63	6.06	0.01	0.93	-32.65
	9	5	78.66	6.08	0.01	0.94	-32.66
	8	5	78.69	6.12	0.01	0.95	-32.68
	22	6	79.12	6.55	0.01	0.96	-31.09
	26	6	79.13	6.55	0.01	0.96	-31.09
	18	6	79.18	6.6	0.01	0.97	-31.12
	21	6	79.34	6.76	0.01	0.98	-31.2
	17	6	79.76	7.18	0	0.98	-31.41
	31	7	79.88	7.31	0	0.98	-29.44
	23	6	79.97	7.39	0	0.99	-31.51
	29	7	80.3	7.72	0	0.99	-29.65
	28	7	80.35	7.78	0	1	-29.68
	20	6	82.08	9.5	0	1	-32.57
	27	7	83.13	10.56	0	1	-31.07
	30	7	83.18	10.61	0	1	-31.09
	32	8	84.23	11.65	0	1	-29.31

Table S3.12: Complete model selection tables from multiple regression analyses to assess the best phenotypic predictors of relative between-lineage fertilization success for Italian males. The top supported models (< 2 ΔAICc) are highlighted.

	Italian Males Relative Between - Lineage Fertilization Success						
	Candidate Model	K	AICc	Delta AICc	AICc Weight	Cumulative Wt	Log Likelihood
Body Size and Performance	1	3	95.07	0	0.29	0.29	-44.06
	5	4	96.74	1.67	0.13	0.42	-43.54
	3	4	97.13	2.06	0.1	0.52	-43.73
	2	4	97.38	2.31	0.09	0.61	-43.86
	4	4	97.53	2.46	0.08	0.69	-43.93
	10	5	97.67	2.59	0.08	0.77	-42.53
	11	5	98.56	3.48	0.05	0.82	-42.97
	8	5	98.6	3.53	0.05	0.87	-43
	6	5	100.03	4.95	0.02	0.9	-43.71
	9	5	100.07	5	0.02	0.92	-43.73
	7	5	100.27	5.2	0.02	0.94	-43.83
	15	6	100.66	5.58	0.02	0.96	-42.42
	13	6	100.73	5.65	0.02	0.98	-42.45
	14	6	101.3	6.23	0.01	0.99	-42.74
	12	6	103.24	8.16	0	1	-43.71
	16	7	104.08	9.01	0	1	-42.37
Colouration	5	4	97.16	0	0.13	0.13	-43.78
	1	3	97.63	0.47	0.1	0.23	-45.35
	10	5	97.75	0.59	0.09	0.32	-42.63
	2	4	97.77	0.61	0.09	0.41	-44.08
	9	5	98.24	1.08	0.07	0.48	-42.87
	6	4	98.37	1.2	0.07	0.55	-44.38
	7	5	99.5	2.34	0.04	0.59	-43.5
	16	5	99.72	2.56	0.04	0.63	-43.61
	8	5	99.83	2.67	0.03	0.66	-43.67
	3	4	99.93	2.77	0.03	0.69	-45.16
	14	5	100.01	2.85	0.03	0.72	-43.75
	19	6	100.05	2.89	0.03	0.75	-42.2
	12	5	100.05	2.89	0.03	0.78	-43.77
	4	4	100.19	3.03	0.03	0.81	-45.29
	22	6	100.33	3.17	0.03	0.84	-42.34
	21	6	100.52	3.36	0.02	0.86	-42.43
	20	6	100.86	3.7	0.02	0.88	-42.6
	18	6	101.05	3.89	0.02	0.9	-42.7
	13	5	101.08	3.92	0.02	0.91	-44.29
	15	5	101.26	4.1	0.02	0.93	-44.38
	17	6	101.8	4.63	0.01	0.94	-43.07
	11	5	102.74	5.58	0.01	0.95	-45.12
	25	6	102.86	5.7	0.01	0.96	-43.6
	26	6	102.86	5.7	0.01	0.97	-43.6
	28	7	103.07	5.91	0.01	0.97	-41.99
	23	6	103.15	5.99	0.01	0.98	-43.75
	29	7	103.27	6.11	0.01	0.98	-42.09
	30	7	103.41	6.25	0.01	0.99	-42.16
	27	7	103.91	6.75	0	0.99	-42.41
	24	6	104.23	7.07	0	1	-44.29
	31	7	106.29	9.13	0	1	-43.6
	32	8	106.64	9.48	0	1	-41.89

Table S3.13: Partitioning of the (co)variance in male reproductive success into within-lineage and between-lineage components. M, N, and P represent the contributions of mating success, mate fecundity and paternity share, respectively.

	ITA			WEUR		
Source	Value	Standardized Value	% of Total	Value	Standardized Value	% of Total
Variance in Fertilization Success	12.3	0.75	100	4.63	0.75	100
Within-Lineage (W)	11.5	0.69	93.1	4.57	1.44	98.8
Between-Lineage (B)	1.16	0.07	9.38	0.35	0.11	7.58
2 × Cov(W,B)	-0.15	-0.02	-2.36	-0.14	-0.09	-6.16
Within-Lineage Terms						
M_W	1.45	0.44	58.8	0.85	3.05	65.9
N_W	0.80	0.02	3.06	1.10	0.15	3.22
P_W	0.07	0.18	23.9	0.03	0.46	10.0
$Cov(M_W,N_W)$	-0.13	-0.01	-1.59	-0.08	-0.02	-1.26
$Cov(M_W,P_W)$	0.03	0.03	3.88	0.00	0.00	0.22
$Cov(N_W,P_W)$	-0.13	-0.03	-4.59	0.00	0.00	-0.01
Between-Lineage Terms						
M_B	0.64	0.07	9.89	0.35	0.43	9.29
N_B	2.57	0.01	0.84	3.11	0.01	0.13
P_B	0.01	0.01	0.83	0.01	0.01	0.11
$Cov(M_B,N_B)$	-0.24	0.00	-0.55	-0.50	-0.01	-0.53
$Cov(M_B,P_B)$	0.00	0.00	-0.12	0.02	0.01	0.37
$Cov(N_B,P_B)$	-0.09	0.00	-0.42	-0.11	0.00	-0.09

Table S3.14: Standardized linear (β_i) and quadratic (γ_{ii}) regression coefficients from single-trait models with relative within-lineage or between-lineage fertilization success as the response variable. Traits were standardized (mean = 0, SD =1) prior to analysis. Standardized SVL was included in all models except for PC1_BodySize to control for variation in size, and enclosure was included as a random effect. Estimated 95% confidence intervals ($CI_{95\%}$) were generated by bootstrapping with 10,000 iterations. Estimates in bold indicate strong directional or quadratic effects ($CI_{95\%}$ excluding zero). The $CI_{95\%}$ values for the quadratic coefficients are reported for the original estimate while γ_{ii} represents double the original estimate.

Lineage	Relative Fertilization Success	Standardized Trait	n	β_i	$CI_{95\%}$	γ_{ii}	$CI_{95\%}$
ITA	Within-Lineage	Dominance	31	0.41	-0.04 0.86	-0.13	-0.46 0.34
		PC1_BodySize	31	-0.23	-0.62 0.16	-0.32	-0.43 0.10
		Bite Force	30	0.06	-0.43 0.52	0.57	-0.17 0.73
		Testes	30	**0.51**	0.12 0.91	-0.10	-0.33 0.23
		Greenness	31	-0.05	-0.46 0.36	**0.92**	0.00 0.90
		Blackness	31	-0.15	-0.55 0.23	-0.12	-0.34 0.21
		OVS Blue Area	31	0.07	-0.35 0.48	**0.59**	0.04 0.54
		OVS Hue	30	**-0.39**	-0.78 -0.01	0.17	-0.29 0.45
		OVS UV Chroma	30	-0.12	-0.55 0.31	-0.16	-0.39 0.22
	Between-Lineage	Dominance	31	-0.11	-0.61 0.41	-0.80	-0.83 0.04
		PC1_BodySize	31	0.17	-0.24 0.58	-0.33	-0.45 0.12
		Bite Force	31	-0.07	-0.57 0.42	0.07	-0.44 0.51
		Testes	30	0.41	-0.03 0.87	-0.48	-0.55 0.06
		Greenness	31	**-0.43**	-0.83 0.00	0.22	-0.37 0.61
		Blackness	31	0.06	-0.38 0.49	0.17	-0.22 0.39
		OVS Blue Area	31	0.02	-0.41 0.47	0.37	-0.10 0.47
		OVS Hue	30	0.36	-0.04 0.78	-0.25	-0.50 0.25
		OVS UV Chroma	30	-0.32	-0.74 0.10	-0.31	-0.46 0.13
WEUR	Within-Lineage	Dominance	32	0.14	-0.20 0.49	0.22	-0.21 0.43
		PC1_BodySize	32	-0.22	-0.56 0.11	-0.36	-0.43 0.07
		Bite Force	32	0.07	-0.31 0.46	**-0.46**	-0.45 -0.01
		Testes	30	0.23	-0.15 0.61	-0.06	-0.30 0.23
		Greenness	32	-0.05	-0.41 0.31	-0.22	-0.65 0.45
		Blackness	32	0.17	-0.12 0.49	0.37	-0.12 0.49
		OVS Blue Area	32	0.04	-0.30 0.38	0.10	-0.16 0.25
		OVS Hue	24	-0.15	-0.60 0.28	**0.60**	0.07 0.52
		OVS UV Chroma	24	-0.34	-0.73 0.03	0.14	-0.39 0.51

3.10 Supplementary Information

Additional information on the quantification of morphological traits

Body Size and Performance

(i) Body Size: for all animals, we collected basic morphometric traits in the field using standard techniques: Snout-vent length (SVL) measured with a ruler to the nearest mm, Body Mass measured to the nearest 0.01 g using digital scales, and Head Length and Head Width measured to the nearest 0.1 mm with callipers.

(ii) Bite Force: prior to release within the enclosures, we measured the bite force of each lizard to the nearest 0.01 Newtons using a specially designed bite force meter (see While et al. 2015 for further details). In brief, the bite force meter was constructed from a modified Sauter FK 25 N force meter with two metal plates on which each animal bites, one attached to the main body of the force meter, and the other attached to the recording rod. Each animal was tested in the middle of the lab light cycle, to maximise the likelihood that they had reached their optimal body temperature. We conducted three successive trials for each individual and retained the largest bite force recording as the representative measure for maximum bite force. To control for any variation in body temperature at the time of testing, we recorded the skin surface temperature of each individual using an infrared duel laser digital thermometer. Body temperature did not predict maximum bite force and was subsequently dropped from all analyses ($\chi^2 = 1.31$, $p = 0.25$).

(iii) Testes Mass*:* following the experiment, we euthanized (Schedule 1 methods: concussion followed by permanent destruction of the brain) and dissected all recaptured males, and removed both testes. We weighed each testis to the nearest 0.001 g using a digital balance and retained the mean mass for analyses.

Colouration

(i) Dorsal Colouration: upon capture, two authors (TU and GMW) scored the lizards for the intensity of their dorsal greenness (Greenness) on a scale of 1 (brown) to 10 (green). Where scores conflicted, we retained the mean value for analyses. In the lab, following their recapture from the experimental enclosures, one author (GPL) used a USB-2000 portable Ocean Optics diode-array spectrometer and a PX-2 xenon strobe light source to perform spectrophotometric measurements on the dorsal surface of each male, outside of the black patterned areas. From each male we obtained a measure of Dorsal Hue, the wavelength at peak reflectance, and Dorsal Green Chroma, the proportion of reflectance within the green spectrum (defined as 496 nm – 570 nm) to that of the entire visible spectrum (defined as 300 nm – 700 nm), denoted $R_{496\text{-}570}/R_{300\text{-}700}$. We confirmed that the Greenness scores were highly correlated objective measures of dorsal colouration (Dorsal Hue: r = - 0.88, Dorsal Green Chroma: r = 0.89), and retained Greenness for regression analyses investigating morphological targets for selection.

(ii) Ventral Blackness: one author (GMW) photographed each lizard on their ventral and left lateral side with a Canon EOS 350D digital camera set with customised white balance (at the beginning of each photography session) and against an X-rite Colour-Checker chart. One author (HEAM) scored ventral blackness from these photographs by quantifying the proportion of black to non-black pixels on each lizard's chest using the program ImageJ (available at http://imagej.nih.gov). To do this, the chest section was highlighted on each individual and the area of black pixels was manually selected using the threshold function. The black area was divided by the total area selected on the chest to generate a proportional score that was retained for analyses. The proportion of black covering the chest area is highly correlated with both the stomach ($r = 0.92$, $p < 0.001$, $n = 42$) and the throat ($r = 0.89$, $p < 0.001$, $n = 42$). Thus, we used the blackness score from the chest area to represent the total ventral proportion of the body.

(iii) Outer ventral scales (OVS) colouration: one author (HEAM) estimated the absolute area of OVS scales with blue colouration on the left lateral side of each individual using photographs taken upon capture and the computer program Image J (Abramoff *et al.*). A scaled object in each photo was used to set the scale for each image and the polygon tool was used to manually trace around the blue areas to estimate the overall area of colouration. For the quantification of OVS Hue and OVS UV Chroma, one author (GPL) took objective colour measurements from the UV-blue OVS of each male, using a USB-2000 portable Ocean Optics diode-array spectrometer and a PX-2 xenon strobe light source (Pérez i de Lanuza *et al.* 2014). Colour measurements were taken from the second and third rostral-most UV-blue OVS on the right and the left flank of each male. From these measurements, we extracted OVS Hue, the wavelength at maximum reflectance and OVS UV Chroma, the proportion of total reflectance in the UV-blue spectrum (defined as 300 nm – 400 nm) to that of the entire visible spectrum, denoted $R_{300\text{-}400}/R_{300\text{-}700}$. We used the average values for the four OVS of each male in analyses. The UV-blue on the OVS of eight Western European males were below the minimum area (2 mm diameter) required for reliable quantification of colour.

Additional information on enclosure assignment

To reduce population of origin effects, for both lineages we released lizards into each enclosure sourced from three different populations. There were two exceptions where the enclosure included Western European females from only two different populations. Within the population of origin constraint, we allocated the lizards to their enclosures at random. As a result, there was overlap in the body sizes of ITA and WEUR males within all eight enclosures (overall SVL range for males: ITA: 52–71 mm; FRA: 51–68 mm). Italian males were on average larger, which reflects natural variation in the wild, however, there was no significant difference in body size between Italian and Western European males in any of the enclosures (LM: Enclosure: $F_{1,7}$ = 0.21, p = 0.98, Origin: $F_{1,1}$ = 3.09, p = 0.09, Enclosure × Origin: $F_{1,7}$ = 0.19, p = 0.99).

Additional information on spatial and behavioural data

(i) Positional data: we collected ~ 79 hours of positional data during the experiment (3rd May to 25th June 2013). During 10 minute observation periods, one observer (HEAM or JB) collected positional data by recording the identity and location of all visible lizards within an enclosure on to a scale map that included the nine pallet habitats. We did this for all eight enclosures in succession (an observation round). On average (since data collection was weather dependent) we performed five observation rounds per day, with ~2 hour intervals (minimum 1.5 hours) between observation rounds. We began taking observations when the first lizards were observed in the morning and ended at dusk. The sequence of enclosures during positional data collection remained the same throughout the experiment; however, we rotated the first enclosure of the round between days to avoid a temporal effect on observations. We only recorded the first location where an individual was observed during a single 10 minute period. We also collected social interactions opportunistically during positional data collection.

(ii) Spatial analyses: positional data were entered into Ranges 8 (Kenward *et al.* 2008). For the generation of kernel, we set the matrix cell number to 40 and the smoothing parameter to 0.75 times the reference smoothing parameter (href, the standard deviation of rescaled x and y coordinates divided by the sixth root of the number of locations). We selected 0.75 × href by visual assessment because it provided the best balance between under and over-smoothing and could be applied to all lizard ranges (see Kie 2013 for similar ad hoc methods).

(iv) Habitat assignment: the kernel centre, the location where the Gaussian kernel estimate indicated peak density, fell directly on a pallet for all but five lizard ranges. We retained the quality of this habitat (High, Medium or Low) for analyses as a representative of each lizard's core home range habitat quality. For the five exceptions, we selected the habitat quality of the nearest pallet.

(vii) Statistical analyses of spatial data: we tested for lineage and sex differences in positional observations, core home range area, and total home range area, using LMMs with Sex, Lineage and their interaction as fixed effects. We tested for an effect of Lineage and Dominance on male-female overlap using GLMMs (Poisson) with Lineage, Dominance and their interaction as fixed effects, and number of overlapping females, number of overlapping same lineage females, and number of overlapping other lineage females as separate response variables (overlap determined from 50% isopleths) . We excluded males with home ranges centred on medium quality habitat (n = 9) from analyses of habitat selection, due to their low number. We tested for an effect of Lineage and Dominance on male habitat quality with a GLMM (Binomial), with high or low habitat quality as the response variable and Dominance, Lineage and their interaction as fixed effects. To test the spread of positional observations, we performed a LMM with Spread as the response variable and Origin, Habitat Quality and their interaction as fixed effects. Enclosure was included as a random effect in all mixed model analyses of spatial data.

(viii) Classification of social interactions: to quantify the number, direction and outcome of interactions between individuals, one observer (HEAM or JB) carried out additional 45 minute observation periods on the enclosures. We recorded the identity of interacting lizards, the initial location of the receiver, and the nature of the social interaction according to an ethogram (Table S3.2). We classified behavioural interactions into three categories: male-male agonistic, male-female courtship and other. Male-male agonistic interactions included behaviours such as chases, physical attacks and aggressive posturing between males. To distinguish these interactions from non-combative male-male behaviour, we only classified interactions as male-male competition when they included a submissive behaviour by one male in the presence of another (i.e. a retreat) and this determined which male was deemed the "winner" of the encounter. We used this outcome to generate male dominance scores (David 1988). We classified male-female interactions as courtships when they included display behaviour /from a

male directed towards a female or a tail grab by the male. We deemed these behaviours indicative of male sexual interest in a female or intention to mate.

(iv) Dominance scores: based on wins and losses during dyadic male-male competitive interactions, we calculated a within-enclosure index of social dominance for each male (David 1988), which we corrected to control for chance and normalised to account for group size after the death of one Italian male from one enclosure at the start of the experiment. Dij based David's Dominance scores for each male were calculated in R package 'Steepness' following Gammell et al. (2003) with correction and normalisation described by de Vries et al. (2006): the corrected dyadic dominance index (Dij) for individual i in his interactions with another individual j is calculated according to the formula $Dij=(Sij+0.5)/(nij+1)$ where Sij is the number of times that i defeats j and nij is the total number of interactions between i and j. For each enclosure, we generated a dyadic dominance index matrix based on Dij. From these matrices, the David's score for each male i of an enclosure was calculated with the formula $DS = (W + W2) - (L – L2)$ where $W = \sum Dij$, $W2 = \sum W$ (weighted by the appropriate Dij values of those individuals with which i interacted), $L = \sum Dji$ and $L2 = \sum L$ (weighted by the appropriate Dji values of those individuals with which i interacted). We then normalized the DS values using $NDS = [DS + (N(N - 1)/2)/N]$ where N is the total number of males in an enclosure (7 or 8). NDS values were used as Dominance scores in subsequent analyses.

Additional information on DNA extraction and paternity analysis

(i) DNA extraction: we isolated DNA from tail-tip tissue samples following QIAGEN DNeasy extraction protocol (Qiagen, Shanghai, China) in a final elution volume of 150µl (in AE buffer). And carried out PCR reactions 6 microsatellite loci (Heathcote *et al.* 2015, Table S3). We combined the primers into two multiplexes: MP1 (C150, C168, C275-278) and MP2 (C164, C038, C028). Each multiplex contained 5 µl of Qiagen Master Mix, 0.2 µl (190 µl dH20:10 µM Primer) of forward and reverse primer and 3.8 µl of PCR grade dH2O. We carried out PCR

reactions on 11 µl reaction volumes (1 µl of DNA template and 10 µl of Multiplex) under the following PCR conditions: 15 min of initialization step at 95°C, 26 cycles of 30 sec at 94 °C, 1:30 min at 57 °C and 1 min at 72 °C and a final extension step of 20 min at 60 °C. The 5'-end of each forward primer was labelled with a fluorescent dye either 6-FAM or HEX. We diluted the PCR products in ddH2O (1:5 dilution) and, together with an internal ladder (Red ROX-500), these were genotyped on an ABI 3130 genetic analyser (Applied Biosystems Inc.). A single author (HEAM) scored the alleles in Geneious 7.0.4 (Biomatters. Available from http://www.geneious.com/). The mean allele frequency per locus was 28.

(ii) Paternity analysis: we performed a simulation paternity analysis based on 100,000 offspring and eight candidate fathers to estimate the critical values of the log-likelihood statistics (LOD scores). We assigned paternity based on the trio (mother, father, offspring) LOD score, using a strict confidence level of 95%, with all eight adult males in each enclosure as candidate fathers. Because 18 offspring mismatched within their trio at more than one locus, we performed a second analysis with all experimental males as candidate fathers. This confirmed that seven of 18 offspring (all of pure Italian origin) were strongly assigned to the cage mate of the female prior to the experiment. This suggested that paternity was the result of sperm storage in these cases (but see Pelliteri-Rosa 2012).

Additional information on variance partitioning

To assess the relative contribution of pre-and post-copulatory sexual selection for net male reproductive success, we further divided the total variances in within-lineage and between-lineage fertilization success into the variance contributions of three components: the total number of genetic partners i.e. females with whom offspring were sired (M), fecundity of those partners i.e. the average number of offspring produced based on genotyped offspring and

embryos (N), and paternity share i.e. the proportion of offspring sired by the male out of the total number of offspring produced by all his partners (P). Males with no mate partners were given a score of zero for M but were excluded from the analyses of N and P. The variances in M, N, and P were then combined within each lineage to produce standardized values and a standardized percentage variance for within-lineage and between-lineage fertilization success (Webster *et al.* 1995; Table S3.13).

Additional information on multiple regression analyses

We tested for multicollinearity among the standardized traits (within-lineage: mean = 0, SD =1) by calculating variance inflation factors (VIFs), which we found to be < 2 in all cases (Table S3.7), confirming that our models were unlikely to be violated by collinearity (Zuur *et al.* 2010). Individuals with missing trait values were removed prior to running the models.

Fieldwork licenses and permits

Direction Régionale de l'Environnement, de l'Aménagement et du Logement (No 2010/DDEA/SEPR/175, No 2010-11, No 11/2012, No 2010-DDEA-SE-105, No 29/2012, No 11/DDTM/657-SERN-NB, No SE-2010-24), Ministero dell'Ambiente e della Tutela del Territorio del Mare – DG Protezione della Natura e del Mare (prot. PNM-2012-2738, prot. 0011511/PNM, prot. PNM-2012-3878, ISRA prot. 14392, 2764/PNM) and Societas Herpetologica Italica (prot. ISPRA 9139 T/-A31).

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Chapter 4

Chemical communication, sexual selection, and introgression in wall lizards

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4.1 Abstract

Animals base many social and reproductive behaviours on information received via communication. Divergence in communication systems should therefore influence the likelihood that individuals from different lineages interbreed, and hence shape the direction and rate of hybridization. However, few species allow for a detailed examination of associations between putative signals, behaviour and reproductive success during secondary contact. Here, we studied the role of chemical communication in hybridization, and its contribution to asymmetric and sexually selected introgression in a secondary contact zone between two lineages of common wall lizard (*Podarcis muralis*). Males of the two lineages differed in the chemical composition of their femoral secretions. Within each lineage, chemical profiles provided information on male secondary sexual characters, but the within-individual repeatability in relative quantities was highly variable among compounds. In experimental contact zones, chemical composition was weakly associated with male reproductive success in both lineages, and did not predict the likelihood of hybridization. Introgression of chemical profiles in a natural hybrid zone resembled that of neutral nuclear genetic markers overall, but one compound (Tocopherol methyl ether) matched closely with the introgression of visual sexual characters. These results imply that associations between male chemical profiles, sexual characters and reproductive success largely reflect transient and environmentally driven effects, and that genetic divergence in chemical composition, for the most part, is selectively neutral. We therefore suggest that the composition of femoral secretions in wall lizards primarily provide information about residency and individual identity, although the introgression of tocopherol methyl ether suggests that single compounds may function as sexually selected signals.

Keywords: Hybrid Zone, Introgression, Pheromones, Olfaction, Femoral Pores

4.2 Introduction

Population divergence in sexual characters used in communication shapes interactions upon secondary contact, with potential evolutionary consequences (West-Eberhard 1983; Panhuis *et al.* 2001). For instance, where one lineage has evolved exaggerated sexual characters favoured by sexual selection, this can result in asymmetric patterns of introgression (e.g. Parsons *et al.* 1993c; Stein & Uy 2006; Baldassarre & Webster 2013). The majority of research on sexually selected introgression has focussed on the role of traits attributed to inter-sexual selection (e.g., female choice; Ryan and Wagner 1987). However, allopatric divergence in traits that primarily function in intra-sexual communication, including colours and morphological features used in competition between males, can also contribute to hybridization and introgression (see Moore 1987; Loehr *et al.* 2008 as examples). For example, if aggression towards divergent male phenotypes is biased or relaxed in males of one or both lineages (e.g. Pauers *et al.* 2008), certain male phenotypes could have an advantage in accessing high quality resources and females. Alternatively, differences in signals may be used to discriminate and avoid males of the other lineage (e.g. Simeonovska-Nikolova 2006). In both cases, divergence in male communication should mediate spatial organisation within hybrid zones and, as a consequence, encounter rates between males and females of different lineages.

Recent studies of vertebrates demonstrate that an evolutionary history of strong intra-sexual selection can cause males of one lineage to be consistently dominant over males of the other lineage, resulting in asymmetric genetic and phenotypic introgression (Owen-Ashley & Butler 2004; While *et al.* 2015). To avoid physical conflict, male-male contests are often resolved through communication (Searcy & Nowicki 2005), hence divergence in signals or cues associated with dominance and resource holding potential could reinforce or mitigate asymmetric introgression. Within this context, the literature on sexually selected introgression has thus far focussed largely on visual and vocal characters. This is unsurprising given that colours and song are considered reliable signals with well-established roles in both female

choice and male-male competition (e.g. Alonso-Alvarez 2004; Abrahams *et al.* 2005; Zeil *et al.* 2006; Hamilton *et al.* 2013). In contrast, the role of chemical communication in mediating patterns of introgression is less clear, despite that chemical communication is taxonomically wide spread and functionally important in reproductive behaviour (Wyatt 2014).

In many species of lizard, males deposit femoral secretions over their home range (Mason & Parker 2010). These secretions are chemically complex and their composition may mediate social interactions, territoriality, and reproduction (e.g. López & Martín 2002; Carazo *et al.* 2007), and ultimately play a key role in determining mating success (Mayerl et al. 2015). Furthermore, it is widely believed that the composition or prevalence of particular compounds have evolved robust associations with other phenotypic characters and hence serve as signals of male health and competitive ability, i.e., function as badges of status (Martín *et al.* 2007; Lopez *et al.* 2009). In Lacertid lizards, for example, the proportions of cholesterol and campesterol have been shown to correlate positively with body size (Lopez *et al.* 2006; Martin & Lopez 2007), and higher proportions of cholesta-5,7-dien-3-ol, ergosterol and waxy esters have been associated with lower parasite loads and higher immune responses (Lopez *et al.* 2006; Martin *et al.* 2008). This has led to the suggestion that divergence in chemical composition is functional, and may contribute to reduced or biased hybridization upon secondary contact (Gabirot *et al.* 2012; Garcia-Roa *et al.* 2016); but direct evidence for this hypothesis is limited. A role for chemical communication in hybridization and introgression has also been inferred from behavioural experiments suggesting that males discriminate con- and hetero-specifics based on chemical cues (e.g. Cooper & Garstka 1987; Martín & López 2006; Gabirot *et al.* 2010), and the observation that hybridization between chemically divergent but sympatric species is rare under natural conditions (Carretero 2008).

We studied the role of chemical communication in male dominance, spatial organisation and hybridization between two lineages of the common wall lizard (*Podarcis muralis*). These

lineages are native to north-central Italy and Western Europe, and have come together in several zones of secondary contact as a result of natural and human-mediated range expansion (While et al. 2015). Phenotypic divergence between lineages is indicative of differences in the strength of sexual selection on morphology, colouration, and behaviour (Heathcote *et al.* 2016; MacGregor *et al.* in press). Hybridization is asymmetric, with evidence for adaptive introgression of visual sexual characters from the dominant Italian lineage into the Western European lineage (While et al. 2015). If chemical communication is also sexually selected, then we predict (i) divergence in chemical characters between the lineages, (ii) consistent associations between chemical composition and male secondary sexual characters and reproductive success, especially in the Italian lineage where sexual selection has been more intense, and (iii) clines in chemical profiles across the contact zone that resemble other sexually selected traits. To test these predictions we first established the extent of divergence in chemical profiles between lineages and associations with other male phenotypic traits. Secondly, we tested experimentally if the compositions of femoral secretions are associated with spatial organisation, reproductive success and hybridization in experimentally replicated zones of secondary contact. Finally, we examined the pattern of introgression of chemical profiles across a zone of secondary contact and tested if they corresponded to the patterns of sexually selected introgression previously demonstrated for morphology and colouration (While *et al.* 2015).

4.3 Materials and Methods

Study species

Common wall lizards, *Podarcis muralis*, are small (45-75 mm snout-vent length), diurnal lizards that inhabit a range of natural and human-modified habitats across southern and central Europe. Intraspecific diversity is high with several genetically and geographically distinct mitochondrial clades (Giovannotti *et al.* 2010; Schulte *et al.* 2012; Salvi *et al.* 2013). The lineages in this study represent two major mitochondrial clades which diverged in glacial refugia approximately 2 million years ago (Gassert *et al.* 2013; Salvi *et al.* 2013a). Here we refer to them as the Italian lineage (ITA, corresponding to the Tuscan subclade *sensu* Schulte *et al.* 2012b) and the Western European lineage (WEUR). As well as being genetically differentiated the Italian and Western European lineages have diverged in morphology and colouration, which has led to pronounced differences in male secondary sexual characters (e.g. relative head size, bite force, testes mass, outer ventral scale UV-blue reflectance, While *et al.* 2015b; MacGregor *et al.* in press).

Chemical sampling and analysis

Chemical sampling and phenotypic measurements

We captured 172 sexually mature males during their first seasonal reproductive episode (April-May) across three consecutive years (2013 to 2015). Sixty four males were captured from populations in Italy and Western Europe (ITA localities: Prato (43°54'N, 11°06'E), Greve di Chianti (43°35'N, 11°19'E) and Colle di Val D'Elsa (43°25'N, 11°06'E)); WEUR Localities: Dinan (48°27'N, 2°02'W), Josselin (47°57'N, 2°32'W), Pontchateau (47°26'N, 2°05'W), Pouzagues (46°47'N, 0°50'E) for use in our enclosure experiment (hereafter referred to as experimental males). One hundred and eight males were captured from sixteen populations in northern Italy (Figure 4.1, Table S4.1) to test for patterns of chemical introgression (hereafter referred to as

cline males). The sixteen populations form a cline across a natural hybrid zone centred, in terms of mtDNA, near Pisa in Tuscany (While et al. 2015).

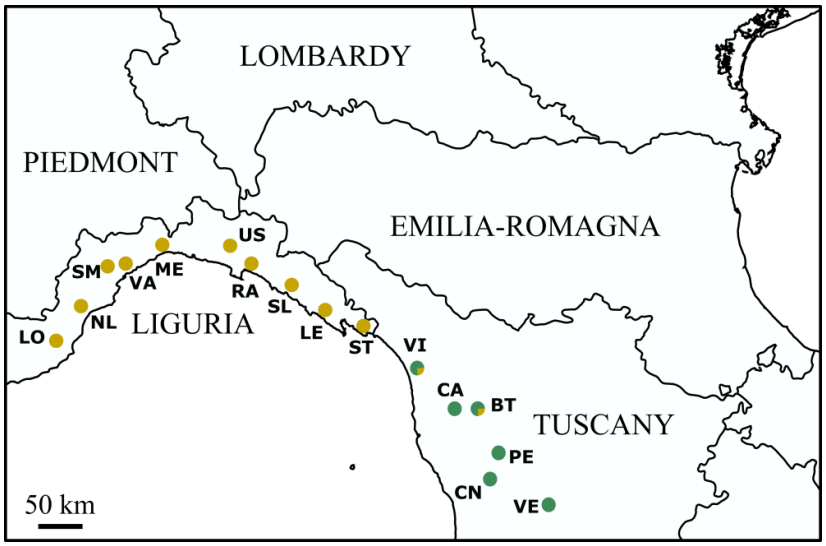

Figure 4.1: Map of the natural contact zone in northern Italy to show the locations of the sixteen populations sampled for cline analyses. Green and brown dots indicate association to the Italian and Western European mitochondrial lineages, respectively (data generated by While et al. 2015). Populations VI and BT have a mix of Italian and Western European haplotypes and approximate the centre of the contact zone.

We collected secretions from the femoral glands of all males by gently pressing around their femoral pores with sterilized forceps. For each male, secretions were collected directly into a glass vial (1.5 mL screw thread vials, Sigma Aldrich). All samples from cline males were collected in the field immediately following capture. For experimental males, we collected two secretion samples from each individual to also assess within-individual variability in chemical composition. The first sample was collected following their capture (April: half of individuals immediately upon capture, and the remaining prior to the release of males into the enclosures, see below), and the second between 49 and 75 days later (in June), immediately following the enclosure experiment. The secretion samples were stored cold while in the field and then at -20 °C until chemical extraction. In addition to femoral secretions we also recorded a number of

morphometric measurements and obtained tissue samples from each lizard for genetic analyses by removing the tip of the tail, which was preserved in 90% ethanol. Morphometric measurements included snout-vent length ((SVL), measured with a ruler to the nearest mm), body mass (measured to the nearest 0.01 g using digital scales), head length and head width (recorded to the nearest 0.1 mm with callipers), ventral blackness and dorsal greenness. From the experimental males we additionally measured testes mass, outer ventral scale colour (OVS blue area, OVS hue and OVS UV chroma), and a performance trait (maximum bite force) in the laboratory (see While et al. 2015; MacGregor et al. in press for full methods regarding morphology data).

Chemical extraction and identification

All secretion samples were dissolved in pentane and analysed by Gas Chromatography Mass Spectrometry (GC-MS) with an Agilent Technologies 7890A gas chromatograph equipped an Agilent HP-5MS capillary column (30m x 0.25 mm x 0.25 μm) with helium as carrier gas at 1mL/min. The oven temperature was programmed at 50 °C for 1 min, increased to 180 °C at 30°C/min, then to 250 °C at 10 °C/min and finally to 320 °C at 5 °C/min and kept at 320 °C for 30 min (total run time per sample = 33.3 minutes). The GC was coupled with an Agilent 5975 C mass spectrometer (MS) with 70eV electron impact ionization.

Where possible we identified chemical compounds within the samples on the basis of their mass spectra (MS) and retention times, which we verified using a computerized MS library (National Institute for Standards and Technology, 2008), and the assistance of an analytical chemist (author ND). Relative retention times were also used to assist in compound identification. When the identity of a compound was uncertain, we added the MS to an "in house" database for recognition across samples. As in previous reports on lizard secretions, including for *Podarcis*

muralis (Pellitteri-Rosa *et al.* 2014), many steroids were not able to be specifically identified. In total, we characterized 67 compounds in the femoral pore secretions of the males (Table S4.2).

To quantify the abundance of each compound we integrated peak areas using MS Data Analysis software (Hewlett-Packard Chemstation Version C.00.07) with fixed integration parameters (Initial Threshold: 16, Initial Peak Width: 0.1, Initial Area Reject: 1.0). Several compounds had similar retention times, and thus co-eluted; so to overcome this we quantified the abundance of fourteen compounds using diagnostic ions selected from high quality spectra (following McLean *et al.* 2012).

From the initial 67 compounds we selected a subset of 42 compounds in the samples of experimental males (Table 4.1). These 42 compounds were commonly occurring (detected in >70% of secretions from the experimental males) and deemed to be reliably quantifiable (either from the Total Ion Current (TIC) or from quantitative ions). For the cline males we selected a subset of 26 compounds to test for patterns of introgression. These compounds were selected because they were consistently occurring (> 93% of experimental males and >99% of males from the contact zone transect). We used different criteria for the selection of compounds between the enclosure males and the cline males because the most variable and transient compounds could mask significant geographic patterns of introgression and thus were deemed inappropriate for the cline analysis. No compounds were lineage-specific; therefore our removal of 16 compounds from the cline analysis was unlikely to exclude potential targets for sexually selected introgression.

For both the experimental and cline males we generated a relative measure of abundance for each compound by log-normal transforming the peak area according to the formula: $Z_{i,j} = ln[Y_{i,j}/g(Y_j)]$, where $Z_{i,j}$ is the standardized peak area *i* for male *j*, $Y_{i,j}$ is the peak area *i* for male *j*, and $g(Y_j)$ is the geometric mean of all peaks for male *j* (Aitchison 1986). To apply the

transformation formula on profiles with non-detectable compounds, we replaced zero values with the proportion of the TIC that represented the minimum percentage detected for a single compound across all samples. Secretion samples showing signs of contamination were excluded (n = 6 experimental samples).

Table 4.1: Details of relative abundance and repeatability for the 42 chemical compounds featuring in the experimental analyses. Compounds are listed in order of their characteristic retention time in minutes (_RT), appended to each compound ID. Detection percentage, and within-lineage mean ($CI_{95\%}$) relative abundance (based on male averages) are reported for each compound, as well repeatability (ICC) estimates ($CI_{95\%}$) based on all males, and separately by lineage. Negative ICC estimates are interpreted and reported as evidence for zero repeatability (Nakagawa & Schielzeth 2010). Compounds in bold differed between the lineages in their relative abundance based on non-overlapping confidence intervals. Compounds highlighted in grey were repeatable based on ICC confidence intervals that excluded zero.

	Detection %	Mean relative abundance ($CI_{95\%}$)		ICC value ($CI_{95\%}$)		
	All Samples	ITA	WEUR	All Males	ITA	WEUR
Heptadecene_RT8.25	100	**-0.03 (-0.32,0.25)**	**0.65 (0.43,0.87)**	0.43 (0.19,0.63)	0.39 (0.03,0.66)	0.25 (-0.15,0.59)
2-Heptadecanone_RT9.85	100	-0.38 (-0.58,-0.18)	-0.39 (-0.61,-0.18)	0.40 (0.15,0.61)	0.42 (0.07,0.68)	0.40 (0.01,0.68)
Palmitic acid (Hexadecanoic acid)_RT10.3	96	1.46 (0.89,2.04)	0.94 (0.30,1.58)	0.02 (-0.25,0.29)	0.35 (-0.01,0.64)	0.00 (-0.66,0.04)
2-Nonadecanone_RT11.5	87	-1.81 (-2.26,-1.37)	-1.25 (-1.70,-0.80)	0.18 (-0.09,0.43)	0.11 (-0.27,0.45)	0.25 (-0.15,0.59)
Oleic acid (9-Octadecenoic acid)_RT11.81	87	0.11 (-0.54,0.76)	0.42 (-0.29,1.13)	0.19 (-0.09,0.43)	0.14 (-0.24,0.48)	0.24 (-0.17,0.58)
Stearic acid (Octadecanoic acid)_RT11.98	90	0.58 (-0.20,1.37)	1.48 (1.01,1.95)	0.10 (-0.17,0.36)	0.23 (-0.15,0.55)	0.00 (-0.55,0.20)
Eicosanoic acid_RT13.7	97	0.01 (-0.36,0.38)	0.75 (0.28,1.22)	0.10 (-0.17,0.36)	0.00 (-0.41,0.32)	0.03 (-0.36,0.42)
Squalene_RT18.34	100	0.97 (0.64,1.30)	0.60 (0.44,0.76)	0.22 (-0.05,0.46)	0.40 (0.04,0.67)	0.00 (-0.62,0.10)
Unidentified_RT18.58	92	-1.60 (-1.88,-1.32)	-1.68 (-2.01,-1.35)	0.00 (-0.28,0.26)	0.13 (-0.24,0.47)	0.00 (-0.48,0.29)
Unidentified Steroid_RT19.1	97	-0.99 (-1.25,-0.74)	-0.89 (-1.02,-0.76)	0.09 (-0.18,0.35)	0.15 (-0.22,0.49)	0.00 (-0.57,0.18)
Cholesta-2,4,6-triene_RT19.22	73	-2.96 (-3.35,-2.57)	-2.52 (-2.92,-2.13)	0.31 (0.04,0.53)	0.21 (-0.16,0.53)	0.42 (0.04,0.70)
Cholesta-3,5-diene_RT19.34	98	-1.08 (-1.31,-0.85)	-0.90 (-1.13,-0.67)	0.20 (-0.07,0.44)	0.38 (0.02,0.66)	0.05 (-0.35,0.43)
Unidentified Steroid_RT19.57	99	0.55 (0.35,0.75)	0.44 (0.24,0.63)	0.09 (-0.18,0.35)	0.26 (-0.11,0.57)	0.00 (-0.43,0.35)
Unidentified Steroid_RT19.75	98	**-0.19 (-0.39,0.01)**	**-0.64 (-0.85,-0.43)**	0.29 (0.02,0.52)	0.26 (-0.11,0.57)	0.23 (-0.18,0.57)
Unidentified Steroid_RT19.86	99	-0.87 (-1.02,-0.72)	-1.07 (-1.26,-0.89)	0.14 (-0.13,0.40)	0.34 (-0.03,0.63)	0.01 (-0.38,0.41)
Unidentified Steroid_RT19.97	96	-0.52 (-0.75,-0.29)	-0.77 (-1.05,-0.49)	0.24 (-0.03,0.48)	0.22 (-0.16,0.54)	0.25 (-0.15,0.59)
Unidentified Steroid_RT20.09	70	**-2.31 (-2.71,-1.92)**	**-3.18 (-3.66,-2.71)**	0.14 (-0.14,0.39)	0.01 (-0.35,0.38)	0.10 (-0.30,0.48)
Unidentified Steroid_RT20.24	73	-2.60 (-2.94,-2.27)	-2.83 (-3.30,-2.36)	0.11 (-0.16,0.37)	0.00 (-0.44,0.29)	0.34 (-0.05,0.65)
Unidentified Steroid_RT20.4	97	-0.96 (-1.16,-0.75)	-1.35 (-1.62,-1.07)	0.46 (0.22,0.65)	0.37 (0.01,0.65)	0.48 (0.11,0.74)
*Unidentified Steroid_RT20.76	100	-0.55 (-0.70,-0.40)	-0.46 (-0.60,-0.32)	0.00 (-0.29,0.25)	0.00 (-0.45,0.27)	0.13 (-0.27,0.50)
***Tocopherol methyl ether_RT20.78**	98	**1.65 (1.30,2.01)**	**-2.04 (-2.44,-1.63)**	0.93 (0.88,0.96)	0.67 (0.40,0.83)	0.74 (0.50,0.88)
Unidentified Steroid_RT20.99	99	-1.28 (-1.49,-1.07)	-1.02 (-1.19,-0.85)	0.39 (0.14,0.60)	0.31 (-0.06,0.61)	0.49 (0.12,0.74)
Unidentified Steroid_RT21.17	93	**-0.35 (-0.78,0.09)**	**-1.44 (-1.84,-1.04)**	0.67 (0.49,0.80)	0.67 (0.40,0.83)	0.56 (0.21,0.78)
***alpha-Tochopherol_RT21.95**	100	**2.69 (2.33,3.05)**	**-1.03 (-1.38,-0.68)**	0.81 (0.69,0.89)	0.31 (-0.06,0.61)	0.37 (-0.03,0.66)
***Cholesterol_RT21.95**	100	**4.65 (4.53,4.77)**	**4.89 (4.78,5.00)**	0.13 (-0.15,0.38)	0.09 (-0.28,0.44)	0.03 (-0.37,0.42)
Cholesta-5,7-dien-3-ol_RT22.27	100	**3.36 (3.20,3.52)**	**2.11 (1.91,2.32)**	0.47 (0.23,0.66)	0.00 (-0.38,0.35)	0.09 (-0.31,0.47)

***Ergosterol (Ergosta-5,7,22-trien-3-ol)_RT22.79**	100	**0.04 (-0.21,0.29)**	**-0.85 (-1.00,-0.71)**	0.31 (0.05,0.54)	0.08 (-0.29,0.44)	0.12 (-0.29,0.49)
***Unidentified Steroid_RT22.95**	100	**-0.45 (-0.86,-0.04)**	**0.49 (0.26,0.71)**	0.32 (0.06,0.54)	0.36 (0.00,0.64)	0.05 (-0.34,0.44)
***Campesterol (Ergost-5-en-3β-ol)_RT23.01**	100	**0.15 (-0.16,0.46)**	**1.35 (1.09,1.61)**	0.36 (0.10,0.57)	0.26 (-0.12,0.57)	0.00 (-0.47,0.31)
*Cholesta-4-en-3-one _RT23.26	100	0.00 (-0.36,0.35)	0.55 (0.20,0.90)	0.37 (0.11,0.58)	0.32 (-0.05,0.61)	0.36 (-0.03,0.66)
***Ergosta-5,8-dien-3-ol_RT23.48**	100	**2.11 (1.96,2.25)**	**1.43 (1.16,1.71)**	0.18 (-0.10,0.43)	0.00 (-0.37,0.35)	0.10 (-0.30,0.47)
*Cholesta-4,6-dien-3-one_RT23.67	96	0.21 (-0.08,0.51)	0.51 (0.18,0.84)	0.00 (-0.40,0.13)	0.00 (-0.50,0.21)	0.00 (-0.51,0.26)
***gamma-Sitosterol_RT24**	92	**-1.09 (-1.57,-0.60)**	**0.22 (-0.12,0.56)**	0.32 (0.05,0.54)	0.17 (-0.21,0.50)	0.28 (-0.13,0.60)
***Stigmastanol_RT24.14**	95	**-0.45 (-0.93,0.03)**	**0.64 (0.28,0.99)**	0.34 (0.08,0.56)	0.26 (-0.11,0.57)	0.27 (-0.14,0.60)
*Unidentified Steroid_RT24.24	99	1.02 (0.83,1.21)	0.67 (0.41,0.94)	0.12 (-0.15,0.38)	0.09 (-0.28,0.44)	0.11 (-0.29,0.48)
***Unidentified Steroid_RT24.48**	99	**2.22 (2.04,2.40)**	**1.46 (1.08,1.83)**	0.38 (0.07,0.55)	0.45 (0.11,0.70)	0.21 (-0.20,0.56)
Unidentified Waxy Ester_RT25.45	100	1.49 (1.22,1.76)	1.91 (1.71,2.11)	0.07 (-0.21,0.33)	0.06 (-0.31,0.42)	0.00 (-0.46,0.32)
Unidentified Waxy Ester_RT25.88	79	0.13 (-0.71,0.98)	0.53 (-0.29,1.34)	0.14 (-0.14,0.39)	0.21 (-0.16,0.54)	0.09 (-0.31,0.47)
Unidentified Waxy Ester_RT26.14	74	**-1.77 (-2.45,-1.10)**	**-0.01 (-0.56,0.54)**	0.08 (-0.19,0.34)	0.00 (-0.39,0.34)	0.05 (-0.35,0.43)
Oleic acid, octadecyl ester_RT28.51	97	**0.18 (-0.12,0.49)**	**1.65 (1.35,1.96)**	0.25 (-0.03,0.48)	0.00 (-0.47,0.25)	0.26 (-0.14,0.59)
Hexadecanoic acid, eicosyl ester_RT28.72	98	**-0.20 (-0.45,0.04)**	**1.04 (0.57,1.52)**	0.30 (0.03,0.53)	0.00 (-0.63,0.02)	0.42 (0.03,0.70)
Unidentified Waxy Ester _RT29.92	81	-1.14 (-1.73,-0.54)	-0.39 (-1.07,0.29)	0.30 (0.03,0.53)	0.10 (-0.28,0.45)	0.50 (0.14,0.75)

* Compounds quantified based on quantitative ions rather than the integration of peak areas

Repeatability and divergence in chemical composition

From the experimental males, we estimated within-individual repeatability in relative abundance of the 42 chemical compounds between first and second secretion samples. Intra-class correlation coefficients (ICC values) and their confidence intervals were calculated using the anova-based method (i.e. Lessells & Boag 1987) implemented in R package, ICC (Wolak *et al.* 2012). Since ICC values were found to be highly variable among compounds (see below), for the subsequent analyses we used the mean relative abundances (based on both samples) to give an overall representation of the chemical profile of each male's secretions during the time of the experiment.

We assessed the extent of chemical divergence between the Italian and Western European lineages using the secretions of the experimental males, which were sourced from allopatric populations to avoid confounding effects of introgression. We performed a principal components analysis (PCA) across both lineages and retained PC1 to PC7 (explaining 73% of the variance) for further analyses (Table S4.3). To test for differences in overall chemical we performed a permutational MANOVA (adonis function, "vegan" package, Oksanen *et al.* 2007) on PC1 to PC7 with lineage as a fixed effect. Chemical divergence between the lineages was visualised by principal coordinates analysis on the relative abundance of all 42 compounds.

Associations with sexual morphology, spatial organisation and reproductive success

We tested experimentally if the composition of femoral secretions could function as sexual signals, via their co-variance with male phenotype, dominance, and within or between-lineage reproductive success, using semi-natural enclosures. In April 2013, we transported 128 sexually mature lizards (the 64 experimental males and 64 females) captured from the allopatric Italian and Western European localities (see above) to laboratory facilities at the Department of Zoology, University of Oxford, UK. The lizards were transported from the field in cloth bags

(kept below 10 °C) and, once in the lab, they were housed in plastic terraria (590 × 390 × 415 mm) under a 12:12 light/dark cycle, and provided with six hours of UV lighting per day prior to the experiment.

Semi-Natural Enclosures Set-up

In May 2013 we simulated the initial stage of secondary contact between the Italian and Western European lineages by releasing lizards into eight (~ 7 × 7m) experimental enclosures at the John Krebs Field Station, University of Oxford. Full details of the experiment are described elsewhere (MacGregor et al. in press). In brief, we released male lizards into one of eight enclosures such that there were four Italian and four Western European males per enclosure. The males were allowed at least nine days to establish territories prior to the release of four Italian and four Western European females per enclosure. We monitored the eight enclosures during May and June 2013 (during the lizard's second seasonal reproductive episode) to collect positional and social interaction data (based on a previously published ethogram, Heathcote *et al.* 2016). To distinguish territorial interactions from non-territorial male-male behaviour, we only classified interactions as male-male competition if they also included a submissive behaviour (i.e. a retreat) by one male in the presence of another. Submissive behaviour determined which male was recorded as the "winner" of the encounter, and this data was used to generate within-enclosure dominance scores for each male (David 1988; Gammell *et al.* 2003).

The core home range area of each lizard was estimated from positional data in Ranges 8 (Kenward *et al.* 2008). We deemed the area of the 50% isopleth, generated using a fixed-kernel contour analysis with a fixed smoothing parameter of 0.75 (a balance between under and over smoothing), to represent a lizard's core home range (see Kie 2013 for similar methods). For each male, we determined the degree to which his core home range overlapped with the core

home ranges of within-lineage males, other lineage males, within-lineage females, and other lineage females by calculating the sum of the percentage of his core range which overlapped. These scores were used as predictors in tests for associations between male chemical profiles and spatial overlap.

At the end of female gestation we recaptured all experimental lizards. Females were housed in terraria until they laid, at which point we removed the clutches and incubated them at a constant 28 °C and humidity (5:1 vermiculite:water volume) until hatching. At hatching, we obtained tail tissue samples from all juveniles for paternity analysis. We isolated DNA from 203 offspring (hatchlings: 191, embryos: 12) and 128 adults using the DNeasy 96 Blood & Tissue Kit (Qiagen), following manufacturer's instructions (with overnight lysis). Given the limited number of potential fathers (eight per enclosure), we genotyped individuals at six microsatellite loci (Heathcote *et al.* 2015), and assigned offspring paternity in Cervus 3.0 (Marshall *et al.* 1998). This resulted in the retainment of 183 offspring for further analyses (see MacGregor *et al.* in press for further details).

Associations of chemical profiles with morphology, behaviour and reproductive success

To enable tests for associations between chemical profiles, male morphology, behaviour and reproductive success, and to assess the putative function of secretions as sexual signals, we performed principal components analyses separately by lineage on the average relative abundances of the 42 compounds. For each lineage, PC1 to PC7 were retained for further analyses (Table S4.4).

To establish the extent to which chemical profiles could function as signals of dominance status and their association with traits linked to male competitive ability, we assessed the strength of correlations between male dominance scores, morphological trait values (standardized within-

lineage: mean = 0, SD = 1) and within-lineage PC scores (Table S4.4, standardized within-lineage: mean = 0, SD = 1). We tested for multivariate relationships between chemical profiles and morphology using MANOVA, firstly with body size and performance related traits (body size (Table S4.5), bite force, testes mass) and secondly with colour traits (greenness, blackness, OVS blue area, OVS hue and OVS UV Chroma), as response variables, and PC1 to PC7 as predictors. To test for statistical associations between chemical profiles and dominance status, we ran a linear mixed model (LMM) for each lineage with male dominance score as the response variable and PC1 to PC7 as predictors. Since dominance depends upon social environment we controlled for enclosure as a random effect.

To examine whether chemical profiles predicted male-male and male-female spatial overlap, we generated candidate LMMs within each lineage, with all possible linear combinations of PC1 to PC7 as putative predictors of overlap (owing to a lack of a priori hypotheses), and enclosure as a random effect. Pairwise interactions between components were not included due to difficulties in their interpretation. We ran and evaluated all candidate models based on second-order Akaike Information Criterion (AICc), and report model-averaged parameter estimates from full-model averaging (ΔAICc $\leq$ 2, Symonds & Moussalli 2011). Multimodal inferences were applied using the R package, "glmulti" (Calcagno & de Mazancourt 2010). We examined associations between chemical profiles and relative within-lineage and between-lineage fertilization success (the latter for Italian males only owing to differences in the incidence of hybridization) following the same method. Relative fertilization success was calculated by dividing the absolute number of sired offspring for a male by the mean of all males within his enclosure.

Patterns of chemical profile introgression across a zone of secondary contact

Cline Analyses

We tested predictions regarding the direction of chemical introgression across our sixteen populations in northern Italy using a geographic cline approach (e.g. Szymura & Barton 1986b; Gay *et al.* 2008a). We first performed a principal component analysis on transformed relative abundances of the 26 selected compounds from the 108 cline samples and retained the first six principal components for further analyses. PC1 to PC6 together accounted for 76% of the total variance in overall chemical profiles (Table S4.6). To test the extent to which geographic variation among populations was a function of isolation-by-distance we performed a Mantel test between a matrix of chemical distances and geographic distances (based on 10,000 permutations). Chemical distances were defined as the mean Euclidean distances among populations based on PC1 to PC6 and geographic distances as linear distances. In addition, we examined the correlation between individual chemical index score and a hybrid index score (generated based on neutral nuclear microsatellite marker for a previous study, While et al. 2015).

To test for patterns of chemical introgression we generated a chemical index from PC1 to PC6 according to the formula: $S = (1 + (DTUS/DSALP))-1$, where DTUS is the Euclidean distance of PCs from an origin defined by the mean PCs of reference Italian individuals (populations VE and PE, Table S4.1), and DSALP is the Euclidean distance from an origin defined by the mean PCs of reference Western European individuals (populations LO, NL and VA, Table S4.1), such that $S > 0.5$ reflects more Italian-like profiles and $S < 0.5$ reflects more Western European-like profiles. Clines were fitted for the chemical index, a hybrid index (for comparison with neutral expectation, While *et al.* 2015b), and male dorsal greenness (for comparison of patterns of selected introgression, While *et al.* 2015b) using the Metropolis-Hastings Markov chain Monte

Carlo algorithm implemented in the package HZAR in R version 3.1.2 (Derryberry *et al.* 2014). For the phenotypic characters we evaluated five candidate models (fitted tails (none, left, right, mirror, or both) all with estimated trait mean and variance (right, left, and centre)), and for the hybrid index we evaluated ten candidate models (all possible combinations of fitted tails (none, left, right, mirror, or both) and scaling (fixed or free) (Derryberry *et al.* 2014). Estimated cline centre and width are reported from the best supported models based on the AIC corrected for small sample size (AICc). The coincidence of cline centres for the chemical index vs. the hybrid index, and for the chemical index vs. greenness were assessed using the maximum-likelihood derived confidence intervals. We additionally performed cline fitting on two putative targets for directional introgression, Tocopherol methyl ether and alpha-Tocopherol, which were selected because of their significant difference in relative abundance between the lineages, high repeatability, and experimental associations with Italian male dominance (see results).

4.4 Results

Chemical composition and consistency

The lipophilic chemical composition of the femoral secretions was consistent with that previously reported for this species (Martin & Lopez 2006a; Martin *et al.* 2008; Pellitteri-Rosa *et al.* 2014), and consisted primarily of steroids (71.3%), but also contained carboxylic acids (11%), their waxy esters (9.2%), Tocopherols (5.8%), Terpenoids (1.4%), alkenes (0.7%) and ketones (0.5%, see Table 4.1 and Table S4.1 for further details). On average the five most abundant compounds across both lineages were cholesterol (47%), cholesta-5,7-dien-3-ol (8.3%), palmitic acid (4.4%), alpha-tochopherol (4.3%) and stearic acid (4.1%). However, the relative quantities of the 42 compounds varied in their within-individual repeatability (range of intra-class correlation coefficients: 0 – 0.9; Table 4.1), suggesting that the femoral secretions contain highly condition-dependent components, but also stable and potentially heritable components (Boake 1989).

Evidence for divergence between the lineages

The overall chemical compositions of secretions differed between the lineages (Lineage: $F_{1,61}$ = 25.77, $p < 0.001$, R^2 = 0.30, Figure 4.2). The lineages differed primarily in the relative abundances of two classes of compounds: tocopherols, which were higher in abundance in the Italian lineage, and waxy esters, which had higher abundances in Western European secretions (Table S4.7). Assessment of the relative abundance of individual compounds showed significant differences in 18 compounds, and of these, 13 compounds had moderate to high repeatability within individuals (ICC confidence intervals that excluded zero, Table 4.1). Because of the strong differences between lineages in the chemical composition of secretions, we continued by examining associations with morphological characters and known (i.e. outer ventral scale

ornamentation) and putative (i.e. dorsal greenness and ventral blackness) colour signals separately within each lineage.

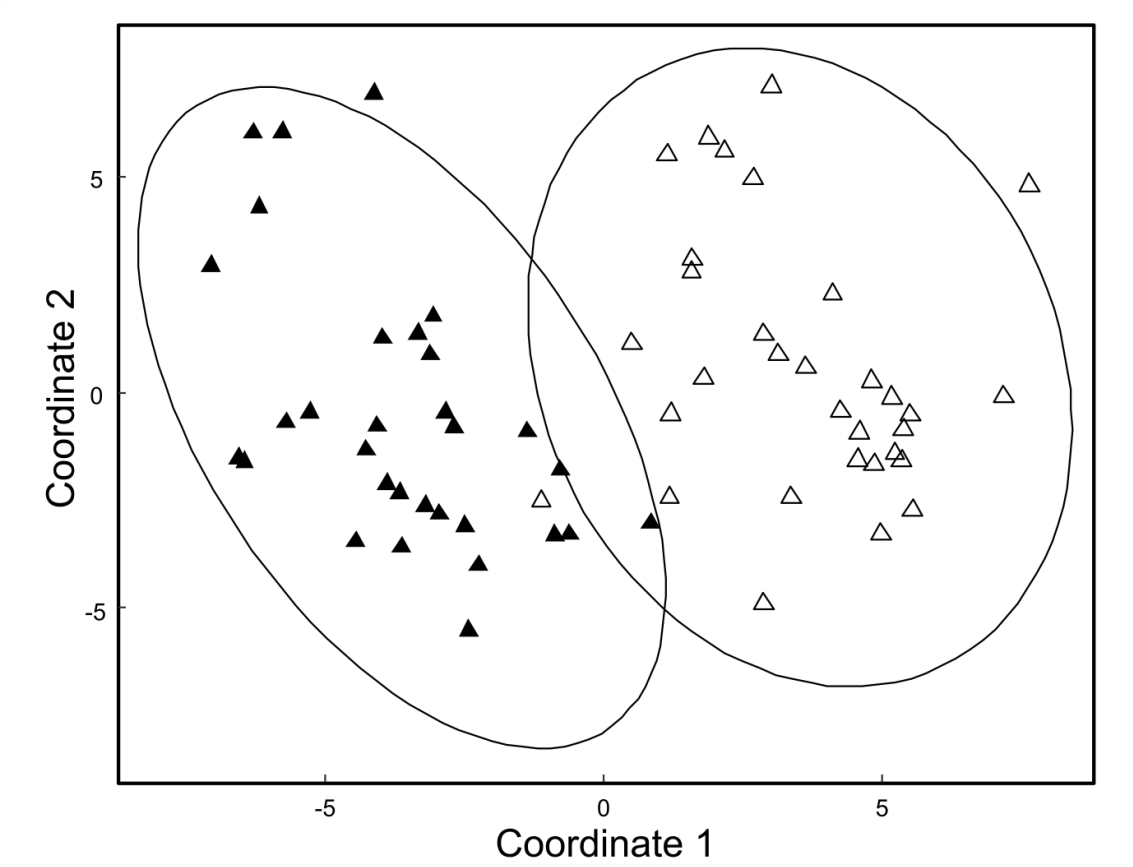

Figure 4.2: Principal coordinates plot of the relative abundance of 42 compounds. Filled triangles are experimental Italian males and unfilled triangles are experimental Western European males. Ellipses represent the 95% confidence for each lineage.

Associations with sexual characters, spatial organisation and reproductive success

Within-lineage chemical variation was correlated with body size, dominance and colouration in the Italian and Western European lineages (Table S4.8). MANOVA revealed that PC6 from both lineages was significantly associated with overall colouration, and, in the Italian lineage, PC1 approached statistical significance in explaining variance in overall body size and performance (excluding dominance, Table S4.9). Chemical variation captured by PC5 was significantly positively associated with dominance in the Italian lineage ($F_{1,23} = 4.58$, $p = 0.043$), and PC6 was significantly negatively associated with dominance in the Western European lineage ($F_{1,19} = 6.32$, $p = 0.021$).

The core territories of Italian males overlapped spatially significantly more with Italian and Western European females, and less with males of their own lineage than Western European males (Figure S4.1). For Italian males several chemical components predicted male-male spatial overlap (PC1, PC4, PC5), however, none were strong predictors of the degree of male-female overlap (Table 4.2). For Western European males chemical profiles predicted male-male (PC1, PC7) and male-female overlap (PC3, PC4, PC6, Table 4.3).

Hybridization was highly asymmetric and occurred mostly between Italian males and Western European females (35% of Western European female offspring sired by an Italian father vs 6% in the opposite direction, reported in MacGregor et al. in press). In the Italian lineage there was some evidence for chemical associations with within and between-lineage reproductive success (i.e. hybridization) however the null mode was equally well supported (Table 4.4). In the Western European lineage, PC4 and PC5 were retained in the top supported models of within-lineage reproductive success, and negatively predicted fertilization success (Table 4.4, association with between-lineage fertilization success in the Western European lineage was not assessed due to the low incidence of hybridization).

Table 4.2: The top supported models (<2 ΔAICc) from analyses to identify associations between chemical profiles (defined as PC1 to PC7, Table S4.4) and male-male and male-female overlap in the Italian lineage. For each model, the number of parameters (*k*), second order Aikaike information criterion (AICc), difference in AICc from the top performing model (ΔAICc), and the relative likelihood of the model (AICcWt) are reported. Model-averaged parameter estimates (model-averaged β) and unconditional 95% confidence intervals (Unconditional $CI_{95\%}$) are also reported (adjacent to predictors on their first appearance in the table), generated via full-model averaging. Parameters with strong effect sizes are in bold.

Overlap	Category	Model	*k*	AICc	ΔAICc	AICcWt	Predictors	Model-averaged β	Unconditional $CI_{95\%}$
Male-Male	Same Lineage	~PC4	4	312.11	0	0.13	PC4	**14.19**	**4.33, 26.01**
		~PC3 + PC4	5	312.20	0.09	0.12	PC3	-8.77	-19.53, 4.45
							PC4		
		~PC1 + PC4	5	312.98	0.86	0.08	PC1	-6.08	-16.80, 5.56
							PC4		
		~PC1 + PC3 + PC4	6	313.09	0.97	0.08	PC1		
							PC3		
							PC4		
	Other Lineage	~PC1 + PC5	5	334.65	0	0.13	PC1	-15.76	-33.93, 4.48
							PC5	19.12	-0.71, 37.62
		~PC5	4	335.46	0.81	0.09	PC5		
		~PC1 + PC5 +PC6	6	336.19	1.54	0.06	PC1		
							PC5		
							PC6	8.33	-9.85, 21.52
Male-Female	Same Lineage	*Null*	3	368.12	0	0.13			
		~PC6	4	369.5	1.38	0.06	PC6	15.00	-18.02, 48.03
		~PC4	4	369.6	1.47	0.06	PC4	14.40	-18.51, 47.30
		~PC1	4	369.85	1.73	0.05	PC1	-12.62	-45.15, 19.91
	Other Lineage	*Null*	3	351.64	0	0.12			
		~PC1	4	351.70	0.06	0.11	PC1	-16.25	-41.57, 9.06
		~PC2	4	0	1.76	0.05	PC2	-9.05	-34.44, 16.33
		~PC1	4	353.59	1.96	0.04	PC1		

Table 4.3: The top supported models (<2 ΔAICc) from analyses to identify associations between chemical profiles (defined as PC1 to PC7, Table S4.4) and male-male and male-female overlap in the Western European lineage. For each model, the number of parameters (*k*), second order Aikaike information criterion (AICc), difference in AICc from the top performing model (ΔAICc), and the relative likelihood of the model (AICcWt) are reported. Model-averaged parameter estimates (model-averaged β) and unconditional 95% confidence intervals (Unconditional $CI_{95\%}$) are also reported (adjacent to predictors on their first appearance in the table), generated via full-model averaging. Parameters with strong effect sizes are in bold.

Overlap	Category	Model	*k*	AICc	ΔAICc	AICcWt	Predictors	Model-averaged β	Unconditional $CI_{95\%}$
Male-Male	Same Lineage	~ PC7	4	340.39	0.00	0.15	PC7	**17.99**	**1.08, 34.89**
		~ PC2 + PC7	5	341.88	1.49	0.07	PC2	-6.91	-22.77, 8.94
							PC7		
		~ PC1 + PC7	5	341.99	1.60	0.07	PC1	7.12	-8.86, 23.09
							PC7		
	Other Lineage	~ PC1	4	336.54	0.00	0.17	PC1	14.28	-1.36, 29.91
		~ PC1 + PC7	5	338.4	1.86	0.07	PC1		
							PC7	4.76	-9.30, 18.82
Male-Female	Same Lineage	~ PC3 + PC6	5	332.68	0.00	0.1	PC3	9.93	-3.23, 23.08
							PC6	-18.01	-35.81, -0.22
		~ PC6	4	333.58	0.90	0.07	PC6		
		~ PC2 + PC3 + PC6	6	333.84	1.16	0.06	PC2	5.56	-6.86, 17.98
							PC3		
							PC6		
		~ PC1 + PC3 + PC6	6	334.58	1.90	0.04	PC1	4.34	-7.59, 16.27
							PC3		
							PC6		
	Other Lineage	~PC4 + PC6	5	348.42	0.00	0.13	PC4	-13.44	-29.23, 2.35
							PC6	**-29.68**	**-52.01, -7.36**
		~ PC1 + PC4 + PC6	6	348.86	0.44	0.1	PC1	-10.57	-27.06, 5.91
							PC4		
							PC6		
		~ PC6	4	349.73	1.31	0.07	PC6		

Table 4.4: The top supported models (<2 ΔAICc) from analyses to identify associations between chemical profiles (defined as PC1 to PC7, Table S4.4) and relative fertilization success within-lineage or between-lineage (Italian males only). For each model, the number of parameters (*k*), second order Aikaike information criterion (AICc), difference in AICc from the top performing model (ΔAICc), and the relative likelihood of the model (AICcWt) are reported. Regression coefficients (β) and 95% confidence intervals ($CI_{95\%}$) are presented for each model. Model-averaged parameter estimates (model-averaged β) and unconditional 95% confidence intervals (Unconditional $CI_{95\%}$) are also reported (adjacent to predictors on their first appearance in the table), generated via full-model averaging.

Lineage	Relative Fertilization Success	Model	*k*	AICc	ΔAICc	AICcWt	Predictors	β	$CI_{95\%}$	Model-averaged β	Unconditional $CI_{95\%}$
ITA	Within-Lineage	~ PC3 + PC6	5	96.44	0.00	0.11	PC3	0.30	-0.05, 0.65	0.09	-0.25, 0.42
							PC6	-0.39	-0.74, -0.05	-0.19	-0.66, 0.28
		~ PC6	4	96.52	0.08	0.10	PC6	-0.39	-0.76, -0.03		
		~ *Null*	3	98.34	1.90	0.04					
	Between-Lineage	~ *Null*	3	102.33	0.00	0.09					
		~ PC2	4	103.19	0.86	0.06	PC2	0.27	-0.13, 0.68	0.10	-0.25, 0.45
		~ PC7	4	103.59	1.26	0.05	PC7	-0.24	-0.65, 0.17	-0.08	-0.39, 0.24
		~ PC6	4	103.78	1.45	0.04	PC6	-0.22	-0.63, 0.18	-0.07	-0.36, 0.23
		~ PC4	4	103.93	1.60	0.04	PC4	-0.21	-0.62, 0.20	-0.06	-0.34, 0.22
		~ PC3	4	104.01	1.68	0.04	PC3	-0.20	-0.61, 0.21	-0.05	-0.33, 0.22
WEUR	Within-Lineage	~ PC4 + PC5	5	91.62	0.00	0.11	PC4	-0.17	-0.32, -0.03	-0.27	-0.68, 0.14
							PC5	-0.17	-0.35, 0.01	-0.16	-0.41, 0.23
		~ PC4	4	92.19	0.57	0.09	PC4	-0.17	-0.33, -0.02		

Patterns of chemical introgression across a zone of secondary contact

Geographic distance between pairs of populations was positively correlated with chemical distance (Mantel Test (10,000 perm): $r = 0.37$, $p < 0.001$, Figure S4.2). Chemical index score was highly correlated with a hybrid index score based on neutral nuclear microsatellite markers generated by While et al. 2015 ($r = 0.75$, Figure S4.3). Consistent with this, cline fitting of the chemical index suggested geographic patterns of chemical variation across the contact zone are similar to that of introgressed nuclear microsatellite markers (Figure 4.3, Table 4.5). From the clines fitted to individual compounds of potential importance, we found that the fitted cline for alpha-tocopherol supported the patterns of neutral introgression (Table 4.5, Figure S4.4). In contrast, our second putative target for selective introgression, tocopherol methyl ether suggested a geographic pattern of variation similar to dorsal greenness (Table 4.5, Figure S4.4).

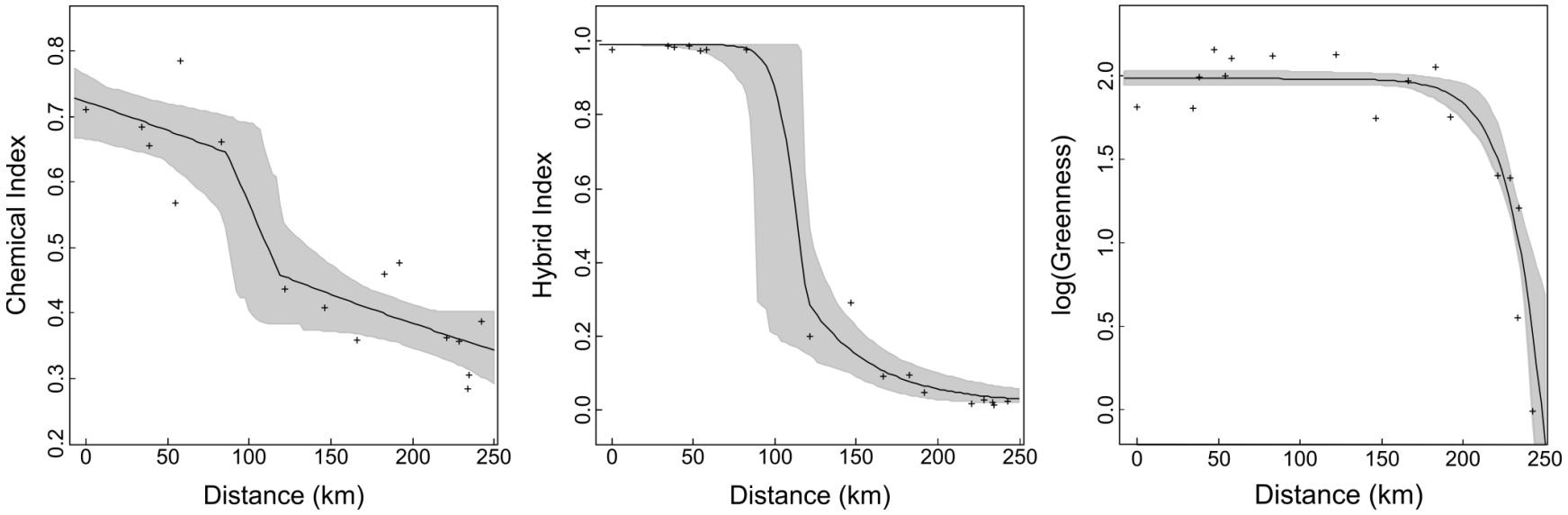

Figure 4.3: The maximum-likelihood cline and the 95% credible cline region for best-fitting models (Table 4.5) for the chemical index (far left), hybrid index (centre) and greenness (scored on a scale of 1–10 and log transformed to improve fit to model assumptions). Transect distance is the cumulative distance from the south-easternmost population Colle di Val D'Elsa (VE) in Tuscany with increasing distance westwards.

Table 4.5: Parameter estimates for best-fitting cline models for the chemical index, hybrid index, and greenness score using HZAR (Derryberry et al. 2014). Parameter c indicates the estimated cline centre (distance from sampling location VE in Tuscany) and w indicates the cline width (1/maximum slope). The parameters pmin and pmax indicate the allele frequencies at the ends of the cline for genetic markers, and δ and τ are exponential decay curve parameters for the left (L) and right (R) tails. Estimated confidence intervals (two log-likelihood unit support limits) are presented in parentheses.

Character	Best Model	c	ω	pmin	pmax	δL	τL	δR	τR
Chemical Index	mirror	99.1 (54.3,139.7)	189.6 (34.0,286.2)						
Hybrid Index	fixed, right	113.9 (99.7,126.2)	48.7 (14.4,84.2)	0	1	NA	NA	61.3	0.4
Greenness	none	273.3 (254.5,279.8)	57.2 (37.2, 88.3)						
Tocopherol methyl ether	none	268.7 (249.2,279.6)	6.4 (-0.1,43.9)						
alpha-Tocopherol	none	113 (83.9,233.8)	42.2 (0.1,241.1)						

4.5 Discussion

Under sexual selection, divergence in chemical signals should mediate patterns of hybridization during secondary contact and, as has been suggested for visual signals, lead to asymmetric patterns of introgression. However, many chemical communication systems function as mechanisms to mediate interactions through individual recognition (Wyatt 2010, 2015). If this is so, chemical composition may not be under consistent selection, and diverge largely due to neutral processes, thereby playing little role in the evolution of reproductive isolation or adaptive introgression.

In this study, we identified characteristics of chemical profiles in two lineages of the common wall lizard. We found that the chemical profiles of wall lizards fulfilled the criteria for sexual signals through their associations with male secondary sexual characters, territorial overlap and reproductive success. However, these associations were variable between lineages and did not predict consistent sexual selection on individual compounds. Furthermore, we found limited evidence for selective introgression of chemical profiles across a natural contact zone where sexually-selected introgression of colour and morphology has previously been documented (While et al. 2015). Combined, our results suggest that divergence in the chemical composition of femoral secretions in wall lizards is largely neutral and that associations with male phenotypes and reproductive success may be transient or environment-dependent and play a minor role in the evolution of reproductive isolation and introgression. Furthermore, this implies that the likely function of wall lizard scent marks is to mediate individual recognition and territory residency rather than to convey physical attributes.

The causes of divergence in the chemical composition of lizard secretions are contentious (Font et al. 2012). It may be driven by differences in the direction and intensity of intra- or inter-

sexual selection on males (Martín Rueda & López Martínez 2014); local adaptation through, for instance, selection for transmission efficiency under differing climates (Alberts 1992; Martin *et al.* 2015); or through adaptively neutral stochastic change (e.g. Runemark *et al.* 2011). Our study goes some way towards testing the sexual selection hypothesis. The two lineages have evolved distinct differences in morphology and visual traits that function in male-male competition, which give a competitive advantage to Italian males. This drives the asymmetric introgression of suites of sexually selected characters from the Italian lineage into the Western European lineage (While et al. 2015). If male chemical profiles have similarly diverged under sexual selection, we would predict that some chemical characteristics associate with male secondary sexual characters; influence success in male-male competition for territory and fertilizations; predict reproductive success and hybridization; and show evidence of adaptive introgression from the Italian to the Western European lineage. In this study, we found evidence for some but not all of these predictions.

The relative abundances of several compounds were associated with sexual characters in both lineages. For example, PC5 (reflecting greater relative proportions of oleic acid, tocopherols, stigmastanol and two waxy esters) was significantly positively associated with male dominance and correlated with colouration (ventral blackness and outer ventral scale UV chroma) in Italian males. There was also a positive association between PC5 and the degree of spatial overlap between the core territories of Italian males and Western European males, which may reflect the fact that Italian males with higher dominance rank tend to be more tolerant of Western European males, the weaker competitors, in their home ranges (MacGregor *et al.* in press). The strongest chemical predictor of the degree of overlap between an Italian male and males of his own lineage was PC4. Italian males with higher values for PC4 had higher proportions of 2-nonadecanone and lower proportions of stigmastanol and waxy esters in their secretions, which were compounds associated with smaller body size, smaller testes mass and less ventral blackness. These males overlapped more with same-lineage males, and less with same-lineage

females, perhaps suggestive of their lower territory holding potential. Similarly, associations with phenotype and spatial organisation were also found in Western European males, although the compounds that predicted sexual morphology or spatial overlap were not always consistent and sometimes even reversed between the lineages.

Despite associations between compounds and sexually selected traits, many compounds showed low repeatability within males and we found only weak associations between the relative composition of a male's secretions and his fertilization success. In the Italian lineage, there was also little evidence that chemical profile predicted hybridization. Furthermore, the best chemical predictors of dominance in the Italian and Western European lineages, PC5 and PC6, respectively, did not feature in the top supported models predicting male fertilization success, despite previous work suggesting that dominance is a strong positive predictor of reproductive success for Italian males (Heathcote et al. 2016; MacGregor et al. in press).

The inconsistencies in both lineages between the chemical characters than associated with dominance and those that were associated with male reproductive success could be partially explained if female preferences influence male mating success, and different chemical characters function in intra-sexual and inter-sexual communication. Indeed, behavioural studies of closely related species suggest females can discriminate between males based on the composition of their femoral secretions (e.g. Lopez *et al.* 2003; Martin & Lopez 2006b). However, a role for inter-sexual selection in shaping the chemosensory traits of wall lizards is not empirically supported by the literature (Font *et al.* 2012b), and, overall, our results are consistent with this conclusion. We found little evidence that females associate with males with a particular chemical composition. Furthermore previous work suggests that neither Italian nor Western European females discriminate based on male quantitative traits or lineage (Heathcote *et al*. 2016).

Based on the weakness of associations between chemical profiles and within- and between-lineage fertilization success we infer that heritable chemical characters that have diverged between the Italian and Western European lineages are unlikely to be under consistent ongoing sexual selection. Indeed, in contrast to morphological and visual traits, the pattern of introgression of chemical profiles conformed to neutral expectations in the presence of asymmetries in hybridization. However, one of our putative targets for selective introgression, tocopherol methyl ether, associated with Italian male dominance, the best predictor of reproductive success in the enclosures, and closely resembled geographic variation in dorsal greenness across the contact zone. Thus direct selection or genetic linkage with genomic regions contributing to sexually selected colour and morphology may cause introgression of this chemical character. Nonetheless, correlations between the hybrid indices (i.e. scores of neutral genetic admixture) and our chemical indices support that overall chemical variation across the contact zone is largely driven by neutral processes.

Presuming that the chemical profile of a male wall lizard's femoral gland secretions is largely not under consistent inter- or intra- sexual selection, what then is the function of chemical communication? One possibility is that the chemical profiles primarily function as a signature mixture, a variable set of compounds which is learnt by other males, allowing them to distinguish individuals (Wyatt 2010, 2014). Indeed, due to their chemical complexity, femoral gland secretions may be better suited than any other cue for use in individual recognition because a very high level of specificity is possible. This explanation is consistent with our observation of only weak associations between male chemical profiles and fertilization success, and is supported by a wealth of empirical studies on lizards demonstrating differential male behavioural responses to the scents of familiar and unfamiliar individuals (e.g. Aragón *et al.* 2001; Font & Desfilis 2002), and even recognition of individual identity based on chemical cues (Carazo *et al.* 2008). If primarily functioning as signature mixtures, the correlations between chemical characters and male sexual characters presented here more likely reflect transient

associations with weakly heritable chemical traits; associations that may easily break down during hybridization, thereby leading to effectively neutral pattern of introgression.

Combined, our experimental and field data highlight the potentially differing functions for visual and chemical communication systems in lizards with consequences for patterns of character introgression between two lineages (see Greig *et al.* 2015 for similar discordant patterns between plumage colour and song in birds). In contrast to recent comparative evidence invoking intra-sexual selection as a mechanism for the evolution of visual traits used for communication in lacertid lizards (Pérez i de Lanuza *et al.* 2013), our study suggests that chemical traits may not be subjected to the same selection pressures. We even suggest that the chemical profiles of femoral gland secretions in wall lizards may not reliably function as sexual signals as is commonly assumed. Instead, the utility of chemical profiles may be because they allow recognition of competitors based on experience.

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4.8 Supplementary Figures

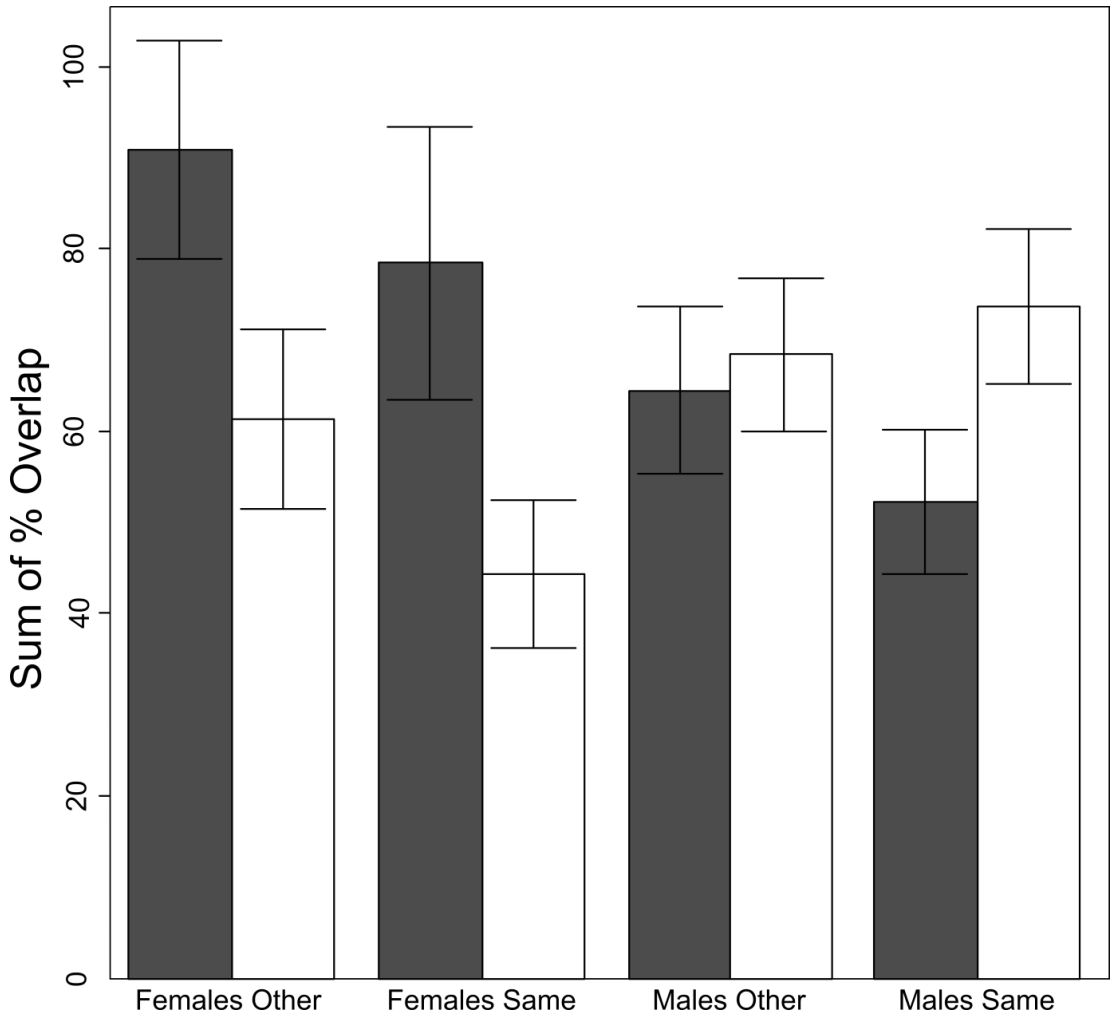

Figure S4.1: Bar plots depicting differences between the lineages in spatial overlap. Italian males (black) overlapped more in core home range with females of both lineages than Western European males (light grey), however, Italian males also overlapped less with males of their own lineage. Each bar represents the mean ± 1 standard error.

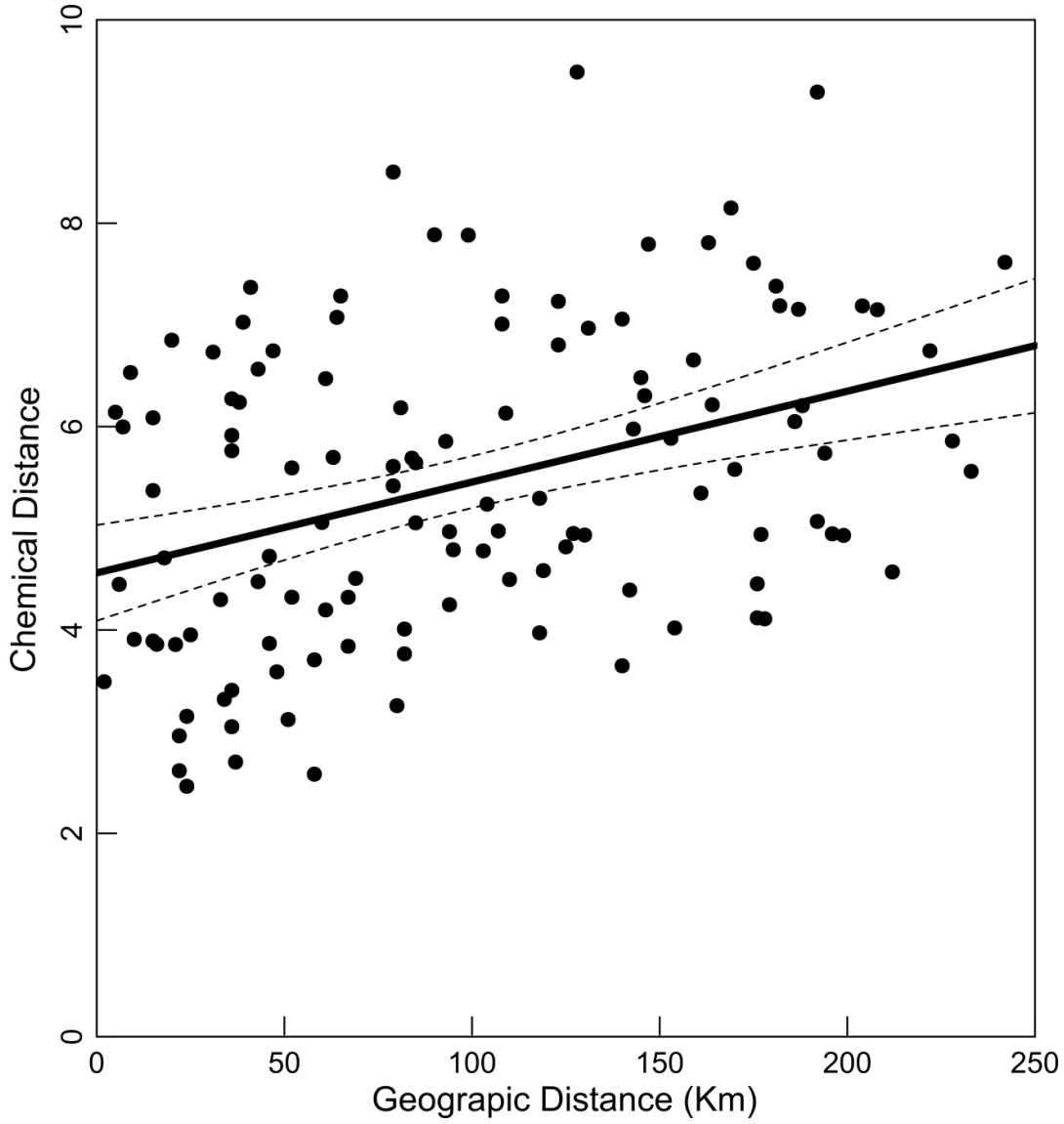

Figure S4.2: The linear relationship between geographic distance and chemical distance among populations (sampled from across the secondary contact zone). Chemical distance was calculated as the mean Euclidean distance between populations based on PC1 to PC6 (see Table S4.6).

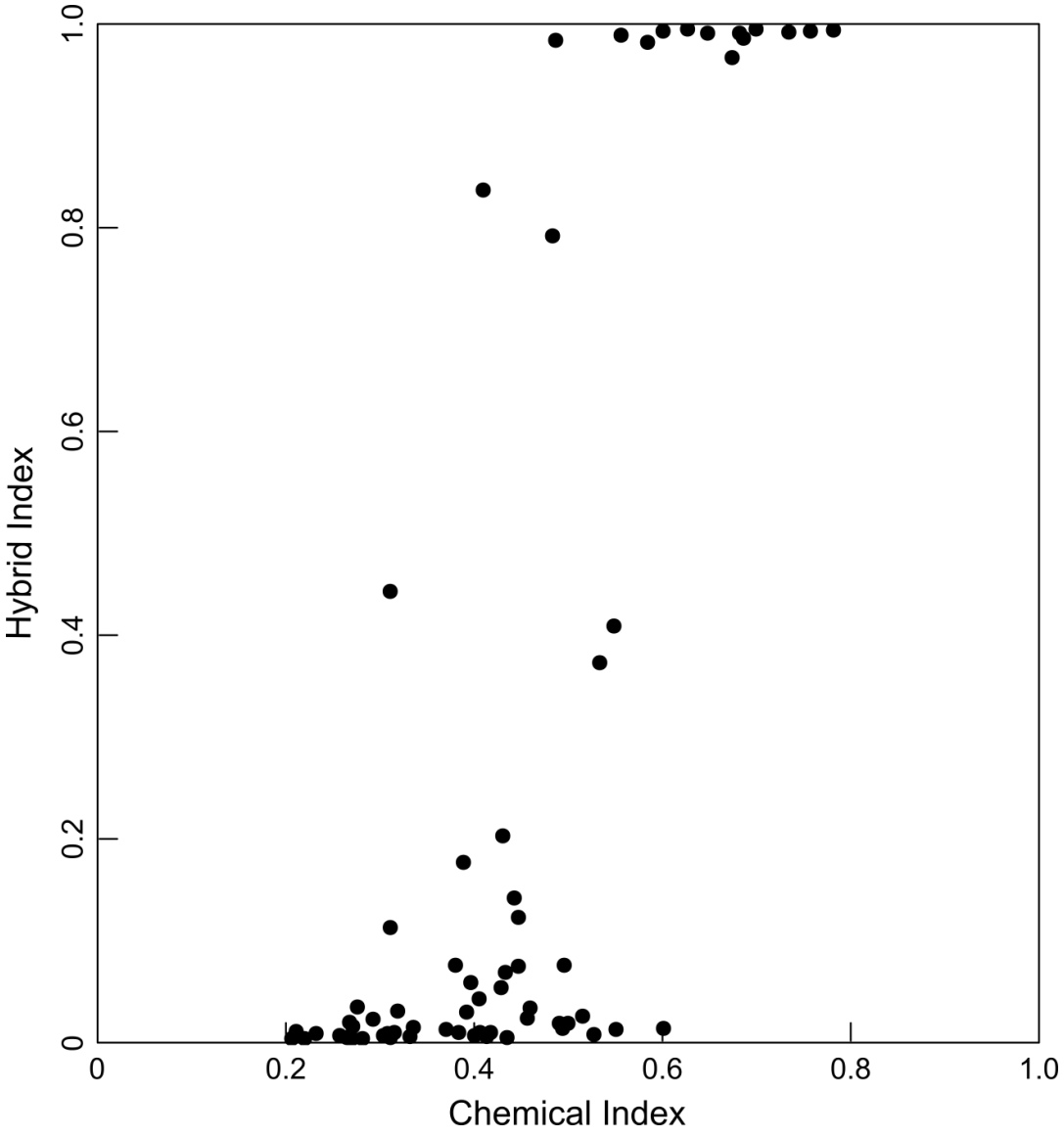

Figure S4.3: The relationship between the chemical index score and hybrid index score for individuals sampled from across the secondary contact zone. Hybrid index scores were generated for a previous study based on neutral nuclear microsatellite markers (While et al. 2015).

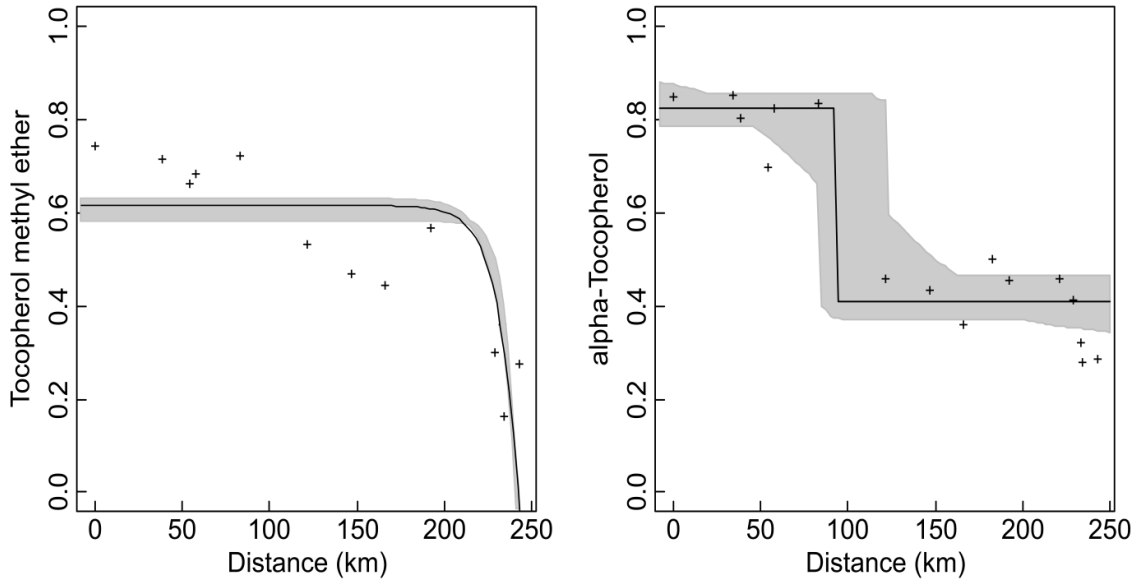

Figure S4.4: The maximum-likelihood cline and the 95% credible cline region for best-fitting models (Table S4.10) for Tocopherol methyl ether and alpha-Tocopherol (data were scaled from 0 to 1 prior to analysis). Transect distance is the cumulative distance from the south-easternmost population Colle di Val D'Elsa (VE) in Tuscany with increasing distance westwards.

4.9 Supplementary Tables

Table S4.1: Population and sampling details in relation to the analysis of patterns of introgression in chemical profiles. Pure Western European (WEUR) and Italian (ITA) reference populations that were used to generate a chemical index are marked in the table under 'Lineage Reference'. Reference WEUR populations are beyond the geographic extent of phenotypic introgression previously reported for sexual morphology (While et al. 2015).

Population	Abbreviation	Latitude	Longitude	Distance to VE (km)	Altitude (m)	Mean Precipitation (mm/month)	Daily Mean Temperature (°C)	mtDNA* Lineage	Secretion Samples	Lineage Reference
Loano	LO	44.13	8.26	242.4	12	92.67	11.96	WEUR	2	WEUR
Noli	NL	44.21	8.41	234.3	7	92.67	11.96	WEUR	6	WEUR
Varazze	VA	44.36	8.58	233.4	5	98.06	9.23	WEUR	9	WEUR
San Martino	SM	44.39	8.52	228.4	322	98.06	9.23	WEUR	6	
Mele	ME	44.44	8.75	220.8	57	98.06	9.23	WEUR	6	
Uscio	US	44.42	9.16	191.7	386	86.39	10.76	WEUR	7	
Rapallo	RA	44.35	9.23	182.5	16	86.39	10.76	WEUR	8	
Sestri Levante	SL	44.27	9.41	166.1	7	86.39	10.76	WEUR	6	
Levanto	LE	44.17	9.61	146.4	12	75.98	11.69	WEUR	10	
San Terenzo	ST	44.08	9.9	121.7	9	75.98	11.69	WEUR	7	
Viareggio	VI	43.84	10.26	82.8	11	73.19	8.75	WEUR,ITA	6	
Calci	CA	43.72	10.52	58.1	40	68.89	10.16	ITA	5	
Buti	BT	43.73	10.59	54.3	94	68.89	10.16	WEUR,ITA	5	
Chianni	CN	43.48	10.64	38.5	297	67.43	12.38	ITA	6	
Peccioli	PE	43.54	10.72	34.4	127	68.89	10.16	ITA	5	ITA
Colle di Val d'Elsa	VE	43.42	11.11	0	229	68.86	9.29	ITA	14	ITA

*Mitochondrial sequences analysed by While et al. 2015. Ecology Letters.

Table S4.2: Details of the 67 lipophilic chemical compounds characterized within the femoral gland secretions of wall lizards. For each compound the characteristic retention time (minutes), mass spectrum ion fragments (M/Z), and, where available, Kovats' Index (see below table for source), are reported.

Label	Compound ID	Chemical Class	Retention Time	Kovats' Index*	Ten largest M/Z ions (% relative intensity to maximum)
Chem1	Heptadecene	Alkene	8.25	1687[a]	83 (100) 97 (92) 55 (82) 69 (75) 57 (73) 70 (54) 56 (49) 111 (49) 71 (42) 84 (40)
Chem2	Tetradecanoic acid	Carboxylic acid	8.83	1748[b]	73 (100) 60 (84) 57 (65) 129 (64) 55 (59) 69 (55) 185 (45) 71 (43) 85 (34) 228 (32)
Chem3	Hexadecanal	Aldehyde	9.19	1795[b]	82 (100) 57 (94) 55 (65) 96 (63) 83 (54) 68 (54) 69 (48) 67 (44) 81 (43) 71 (43)
Chem4	2-Heptadecanone	Ketone	9.85	1875[c]	58 (100) 59 (75) 71 (47) 180 (22) 55 (22) 57 (20) 70 (19) 85 (17) 96 (12) 69 (11)
Chem5	Hexadecenoic acid, methyl ester	Carboxylic acid	10.02	1908[d]	74 (100) 87 (70) 75 (21) 55 (20) 143 (20) 69 (14) 227 (14) 57 (13) 270 (12) 239 (86)
Chem6	cis-9-Hexadecenoic acid	Carboxylic acid	10.24		55 (100) 69 (86) 83 (67) 97 (54) 84 (46) 96 (38) 98 (37) 67 (35) 81 (33) 56 (33)
Chem7	Palmitic acid (Hexadecanoic acid)	Carboxylic acid	10.30	1942[b]	73 (100) 60 (70) 129 (59) 57 (57) 256 (564) 55 (53) 213 (41) 71 (39) 69 (36) 83 (29)
Chem8	Unidentified	Unidentified	11.30		83 (100) 97 (96) 55 (85) 57 (82) 69 (79) 70 (58) 111 (47) 56 (47) 84 (44) 71 (43)
Chem9	2-Nonadecanone	Ketone	11.50	2087[e]	58 (100) 59 (73) 71 (51) 55 (40) 57 (34) 97 (30) 85 (29) 69 (25) 83 (24) 73 (21)
Chem10	Linoleic acid (9,12-Octadecadienoic acid)	Carboxylic acid	11.78	2095[f]	67 (100) 81 (92) 95 (67) 82 (63) 55 (61) 68 (50) 96 (48) 54 (36) 69 (35) 79 (35)
Chem11	Oleic acid (9-Octadecenoic acid)	Carboxylic acid	11.81	2113[g]	55 (100) 69 (91) 83 (85) 97 (78) 84 (52) 98 (46) 70 (43) 57 (40) 111 (40) 56 (36)
Chem12	Stearic acid (Octadecanoic acid)	Carboxylic acid	11.98	2187[h]	73 (100) 60 (72) 57 (71) 55 (64) 129 (62) 284 (60) 71 (46) 69 (46) 83 (40) 185 (35)
Chem13	Unidentified	Unidentified	13.00		83 (100) 97 (96) 69 (78) 57 (71) 55 (70) 111 (50) 82 (45) 70 (42) 71 (37) 56 (35)
Chem14	cis-11-Eicosenoic acid	Carboxylic acid	13.50	2357[i]	55 (100) 69 (82) 83 (73) 97 (72) 57 (52) 81 (43) 67 (41) 84 (41) 96 (40) 70 (36)
Chem15	Eicosanoic acid	Carboxylic acid	13.70	2359[j]	59 (100) 72 (75) 55 (58) 73 (48) 57 (42) 60 (40) 69 (35) 312 (28) 83 (28) 97 (26)
Chem16	Squalene	Terpenoid	18.34	2818[g]	69 (100) 81 (59) 137 (17) 95 (17) 136 (16) 121 (14) 68 (12) 123 (11) 93 (11) 149 (10)
Chem17	Unidentified	Unidentified	18.58		69 (100) 93 (68) 81 (58) 107 (44) 55 (35) 135 (28) 79 (28) 95 (22) 134 (21) 109 (21)
Chem18	Unidentified Steroid	Steroid	19.10		141 (100) 156 (73) 364 (56) 209 (34) 155 (32) 157 (27) 142 (21) 197 (21) 195 (18) 167 (17)
Chem19	Cholesta-2,4,6-triene	Steroid	19.22		135 (100) 366 (90) 143 (77) 119 (61) 141 (49) 149 (46) 95 (46) 129 (45) 81 (45) 157 (43)
Chem20	Cholesta-3,5-diene	Steroid	19.34	2880[h]	368 (100) 147 (85) 145 (61) 81 (56) 105 (53) 107 (49) 95 (44) 353 (39) 93 (38) 91 (36)
Chem21	Unidentified Steroid	Steroid	19.57		251 (100) 364 (76) 197 (49) 155 (26) 105 (24) 349 (24) 365 (23) 252 (21) 141 (17) 159 (12)
Chem22	Unidentified Steroid	Steroid	19.64		69 (100) 81 (93) 71 (42) 135 (28) 95 (27) 93 (27) 121 (20) 109 (20) 107 (19) 68 (18)
Chem23	Unidentified Steroid	Steroid	19.75		364 (100) 195 (67) 209 (65) 197 (36) 349 (34) 365 (30) 181 (28) 179 (22) 165 (20) 196 (19)
Chem24	Unidentified Steroid	Steroid	19.86		69 (100) 81 (93) 71 (42) 135 (28) 95 (27) 93 (27) 121 (20) 109 (20) 107 (19) 68 (18)
Chem25	Unidentified Steroid	Steroid	19.97		350 (100) 195 (68) 366 (48) 183 (43) 143 (42) 351 (37) 141 (33) 158 (31) 210 (25) 196 (20)
Chem26	Unidentified Steroid	Steroid	20.09		251 (100) 364 (91) 235 (61) 197 (52) 362 (51) 141 (44) 155 (39) 376 (37) 195 (33) 249 (32)
Chem27	Unidentified Steroid	Steroid	20.24		141 (100) 156 (73) 378 (54) 155 (33) 209 (29) 157 (27) 195 (19) 142 (19) 197 (18) 379 (17)
Chem28	Unidentified Steroid	Steroid	20.40		141 (100) 156 (79) 364 (57) 155 (35) 209 (32) 157 (26) 142 (20) 197 (20) 179 (19) 365 (19)
Chem29	Unidentified Steroid	Steroid	20.76		251 (100) 378 (71) 197 (54) 105 (27) 379 (22) 155 (22) 252 (21) 363 (19) 141 (18) 179 (17)
Chem30	Tocopherol methyl ether	Tocopherol	20.78		444 (100) 179 (100) 445 (29) 178 (17) 378 (12) 180 (12) 251 (10) 219 (9) 135 (6) 165 (6)
Chem31	Unidentified Steroid	Steroid	20.99		378 (100) 195 (60) 209 (60) 197 (33) 379 (32) 363 (30) 181 (23) 179 (23) 165 (18) 183 (18)
Chem32	Unidentified Steroid	Steroid	21.17		364 (100) 195 (65) 183 (42) 141 (40) 365 (34) 380 (32) 143 (30) 378 (29) 207 (28) 196 (23)
Chem33	Tetracosahexaen-3-ol, hexamethyl (Squalene related)	Terpenoid	21.56		69 (100) 81 (69) 95 (43) 93 (35) 107 (27) 121 (21) 68 (20) 135 (18) 109 (18) 136 (17)
Chem34	Unidentified	Unidentified	21.74		69 (100) 83 (26) 93 (26) 107 (16) 95 (15) 121 (15) 109 (14) 81 (14) 135 (130 105 (11)
Chem35	alpha-Tochopherol	Tocopherol	21.95		165 (100) 430 (80) 164 (32) 431 (25) 166 (12) 205 (11) 57 (8) 121 (6) 55 (6) 432 (4)
Chem36	Cholesterol	Steroid	21.95	3004[i]	386 (100) 275 (66) 301 (59) 368 (50) 353 (43) 145 (41) 213 (39) 105 (38) 107 (38) 371 (37)
Chem37	Cholesta-5,7-dien-3-ol	Steroid	22.27	3160[j]	351 (100) 384 (50) 325 (38) 145 (37) 143 (36) 352 (28) 157 (22) 159 (20) 171 (18) 119 (17)
Chem38	Unidentified Steroid	Steroid	22.57		237 (100) 213 (94) 183 (77) 368 (55) 201 (53) 195 (42) 350 (33) 141 (32) 211 (29) 210 (28)
Chem39	Ergosterol (Ergosta-5,7,22-trien-3-ol)	Steroid	22.79	3087[k]	363 (100) 396 (63) 69 (40) 143 (32) 337 (31) 55 (29) 364 (29) 157 (25) 145 (24) 211 (20)

Chem40	Unidentified Steroid	Steroid	22.95		314 (100) 271 (51) 69 (38) 105 (36) 107 (36) 145 (34) 299 (34) 55 (33) 229 (31) 81 (30)
Chem41	Campesterol (Ergost-5-en-3β-ol)	Steroid	23.01	3193[l]	400 (100) 315 (58) 289 (57) 107 (51) 145 (48) 382 (46) 105 (46) 213 (46) 95 (45) 81 (38)
Chem42	Cholesta-4-en-3-one	Steroid	23.26	3123[m]	124 (100) 229 (51) 384 (35) 261 (27) 135 (20) 95 (20) 107 (20) 147 (20) 260 (19) 149 (18)
Chem43	Ergosta-5,8-dien-3-ol	Steroid	23.48		365 (100) 398 (52) 339 (39) 143 (36) 145 (32) 366 (30) 157 (22) 159 (19) 171 (18) 211 (2)
Chem44	Cholesta-4,6-dien-3-one	Steroid	23.67	3161[n]	382 (100) 338 (89) 136 (56) 269 (47) 215 (39) 227 (35) 131 (35) 198 (35) 95 (35) 160 (33)
Chem45	Unidentified Steroid	Steroid	23.79		237 (100) 213 (97) 183 (86) 201 (56) 382 (53) 195 (44) 364 (32) 210 (28) 155 (25) 211 (24)
Chem46	Unidentified Steroid	Steroid	23.91		414 (100) 147 (63) 412 (58) 135 (54) 379 (52) 105 (44) 133 (40) 91 (39) 95 (38) 119 (36)
Chem47	gamma-Sitosterol	Steroid	24.00	3066[o]	414 (100) 329 (56) 303 (53) 213 (47) 145 (45) 107 (45) 105 (44) 396 (40) 95 (40) 55 (39)
Chem48	Stigmastanol	Steroid	24.14	3310[j]	215 (100) 233 (85) 416 (72) 234 (60) 165 (48) 107 (44) 216 (42) 95 (39) 81 (37) 401 (37)
Chem49	Unidentified Steroid	Steroid	24.24		267 (100) 380 (41) 268 (36) 214 (26) 365 (14) 381 (12) 226 (12) 213 (11) 253 (11) 242 (11)
Chem50	Unidentified Steroid	Steroid	24.38		412 (100) 69 (81) 135 (67) 397 (45) 95 (42) 109 (41) 105 (35) 147 (34) 119 (34) 107 (33)
Chem51	Unidentified Steroid	Steroid	24.48		379 (100) 412 (56) 353 (38) 143 (32) 145 (31) 380 (31) 157 (21) 159 (18) 413 (17) 158 (17)
Chem52	Unidentified Steroid	Steroid	24.65		414 (100) 255 (75) 145 (33) 399 (32) 55 (31) 377 (31) 415 (31) 105 (30) 107 (30) 95 (30)
Chem53	Unidentified Steroid	Steroid	24.76		237 (100) 213 (91) 183 (72) 201 (58) 396 (53) 195 (41) 378 (34) 210 (28) 214 (25) 228 (21)
Chem54	Unidentified Steroid	Steroid	24.92		396 (100) 352 (93) 136 (50) 269 (40) 215 (32) 397 (31) 131 (29) 160 (29) 133 (28) 171 (28)
Chem55	Unidentified Steroid	Steroid	25.02		400 (100) 137 (84) 245 (67) 81 (44) 287 (42) 55 (41) 69 (39) 107 (38) 109 (36) 398 (36)
Chem56	Unidentified Steroid	Steroid	25.18		69 (100) 123 (46) 93 (29) 81 (29) 107 (24) 109 (20) 149 (18) 95 (18) 105 (16) 121 (16)
Chem57	Unidentified Waxy Ester	Waxy Ester	25.45		267 (100) 394 (48) 268 (37) 214 (27) 226 (15) 395 (14) 165 (13) 379 (12) 253 (11) 242 (10)
Chem58	Unidentified Waxy Ester	Waxy Ester	25.88		264 (100) 57 (91) 97 (87) 69 (86) 83 (82) 55 (82) 71 (68) 85 (52) 98 (43) 96 (37)
Chem59	Unidentified Waxy Ester	Waxy Ester	26.14		57 (100) 264 (90) 69 (74) 97 (66) 83 (65) 71 (60) 81 (44) 84 (42) 111 (42) 85 (38)
Chem60	Hexadecanoic acid, octadecyl ester	Waxy Ester	26.34	3546[p]	257 (100) 57 (69) 97 (46) 83 (42) 71 (40) 55 (40) 69 (38) 229 (29) 85 (27) 111 (25)
Chem61	Unidentified Waxy Ester	Waxy Ester	26.41		267 (100) 408 (52) 268 (42) 207 (34) 214 (30) 409 (17) 281 (15) 226 (15) 253 (13) 55 (12)
Chem62	Oleic acid, octadecyl ester	Waxy Ester	28.51		264 (100) 57 (91) 55 (80) 83 (76) 69 (76) 97 (73) 71 (59) 96 (42) 111 (41) 98 (39)
Chem63	Unidentified Waxy Ester	Waxy Ester	28.59		83 (100) 97 (84) 57 (80) 264 (79) 55 (75) 69 (69) 71 (61) 96 (61) 82 (52) 98 (49)
Chem64	Hexadecanoic acid, eicosyl ester	Waxy Ester	28.72		257 (100) 57 (47) 83 (46) 97 (38) 55 (36) 71 (33) 69 (32) 85 (26) 536 (24) 256 (21)
Chem65	Unidentified Waxy Ester	Waxy Ester	29.30		215 (100) 398 (90) 216 (51) 383 (20) 147 (35) 81 (34) 107 (34) 95 (30) 399 (30) 93 (29)
Chem66	Unidentified Waxy Ester	Waxy Ester	29.92		264 (100) 57 (85) 55 (85) 83 (72) 97 (69) 69 (60) 71 (45) 98 (44) 85 (38) 111 (37)
Chem67	Unidentified Waxy Ester	Waxy Ester	30.17		257 (100) 57 (75) 71 (49) 97 (47) 83 (46) 55 (44) 69 (35) 85 (31) 111 (29) 82 (25)

**Source for Kovats' Retention Indices: a, Beens, J.; Tijssen, R.; Blomberg, J., Prediction of comprehensive two-dimensional gas chromatographic separations. A theoretical and practical exercise, J. Chromatogr. A, 822, 1998, 233-251; b, Paolini, J.; Muselli, A.; Bernardini, A.-F.; Bighelli, A.; Casanova, J.; Costa, J., Thymol derivatives from essential oil of Doronicum corsicum L., Flavour Fragr. J., 22, 2007, 479-487; c, Senatore, F., Rigano, D., de Fusco, R., Bruno, M., Volatile components of Centaurea cineraria L. subsp. umbrosa (lacaita) Pign. and Centaurea napifolia L. (Asteraceae), two species growing wild in Sicily, Flavour Fragr. J., 18, 2003, 248-251; d, Blagojevic, P., Radulovic, N., Palic, R., Stojanovic, G., Chemical composition of the essential oils of Serbian wild-growing Srtemisia absinthium and Artemisia vulgaris, J. Agric. Food Chem., 54, 2006, 4780-4789. e, Didaoui, L., Touabet, A., Meklati, B.Y., Comparison of mathematical methods for the calculation of retention indices at high temperature in gas chromatography, J. Hi. Res. Chromatogr., 20, 1997, 605-610. f, Ziegenbein, F.C., Hanssen, H.-P., König, W.A., Secondary metabolites from Ganoderma lucidum and Spongiporus leucomallellus, Phytochemistry, 67, 2006, 202-211. G, Richmond, R., Pombo-Villar, E., Short communication. Use of persistent trace gas chromatography artifacts for the calculation of pseudo-Sadtler retention indices, J. Chromatogr. A, 811, 1998, 241-245. h, Rezazadeh, S., Hamedani, M.P., Dowlatabadi, R., Yazdani, D., Shafiee, A., Chemical composition of the essential oils of Stachys schtschegleevii Sosn. and Stachys balansae Boiss & Kotschy from Iran, Flavour Fragr. J., 21, 2006, 290-293. I, Tret'yakov, K.V., Retention Data. NIST Mass Spectrometry Data Center., 2007. j, Vendramini, A.L., Trugo, L.C., Chemical composition of acerola fruit (Malpighia punicifolia L.) at three stages of maturity, Food Chem., 71, 2000, 195-198. k, Tokuda, H., Saitoh, E., Kimura, Y., Takano, S., Automated analysis of various compounds with a wide range of boiling points by capillary gas chromatgraphy based on retention indices, J. Chromatogr., 454, 1988, 109-120; l, Shlyakhov, A.F., Gas chromatography in organic geochemistry, Nedra, Moscow, 1984, 221.*

Table S4.3: Rotated factor loadings for PC1 to PC7 from a principal components analyses performed on the mean (for a male) relative abundance of 42 compounds. Compounds are listed in order of their characteristic retention time in minutes (_RT), appended to each compound ID. PC1 to PC7 were used for the assessment of chemical divergence between the Italian and Western European lineages. Bold values indicate factor loadings considered strong (> |0.20|).

Label	Compound ID	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Chem1	Heptadecene_RT8.25	-0.11	-0.06	0.10	0.00	0.07	-0.17	0.01
Chem4	2-Heptadecanone_RT9.85	-0.03	-0.09	0.09	0.00	0.04	-0.15	-0.01
Chem7	Palmitic acid (Hexadecanoic acid)_RT10.3	0.00	**-0.49**	-0.08	-0.06	-0.17	-0.02	-0.05
Chem9	2-Nonadecanone_RT11.5	-0.11	-0.15	0.07	0.04	0.12	**-0.27**	0.09
Chem11	Oleic acid (9-Octadecenoic acid)_RT11.81	-0.14	**-0.51**	0.04	-0.16	0.03	**0.32**	0.02
Chem12	Stearic acid (Octadecanoic acid)_RT11.98	**-0.21**	**-0.45**	-0.08	-0.02	-0.17	-0.01	-0.01
Chem15	Eicosanoic acid_RT13.7	-0.10	0.01	0.18	**0.30**	0.08	-0.02	-0.15
Chem16	Squalene_RT18.34	0.05	-0.04	0.01	-0.05	-0.04	-0.17	0.04
Chem17	Unidentified_RT18.58	0.00	-0.01	0.03	0.01	**0.22**	-0.05	-0.12
Chem18	Unidentified Steroid_RT19.1	0.00	0.02	-0.04	-0.06	0.15	0.01	0.12
Chem19	Cholesta-2,4,6-triene_RT19.22	-0.07	0.02	-0.05	-0.10	0.03	**0.22**	0.04
Chem20	Cholesta-3,5-diene_RT19.34	-0.04	-0.03	0.05	-0.07	0.14	-0.15	-0.09
Chem21	Unidentified Steroid_RT19.57	0.04	0.05	-0.07	-0.09	0.09	-0.02	0.11
Chem23	Unidentified Steroid_RT19.75	0.08	0.02	-0.09	-0.12	0.12	0.07	0.10
Chem24	Unidentified Steroid_RT19.86	0.05	0.06	-0.03	-0.05	0.09	-0.03	0.08
Chem25	Unidentified Steroid_RT19.97	0.05	0.06	-0.13	-0.17	0.09	0.04	0.17
Chem26	Unidentified Steroid_RT20.09	0.12	0.04	0.12	0.15	**0.25**	0.03	**-0.38**
Chem27	Unidentified Steroid_RT20.24	0.08	0.08	-0.07	0.19	0.14	**0.39**	0.12
Chem28	Unidentified Steroid_RT20.4	0.07	0.03	-0.10	-0.14	0.06	0.05	0.13
Chem29	Unidentified Steroid_RT20.76	0.00	0.02	0.00	0.01	0.04	-0.08	0.08
Chem30	Tocopherol methyl ether_RT20.78	**0.50**	-0.10	0.08	-0.04	**-0.29**	-0.09	-0.05
Chem31	Unidentified Steroid_RT20.99	-0.02	0.04	-0.04	0.10	0.06	0.04	0.08
Chem32	Unidentified Steroid_RT21.17	0.18	0.07	-0.13	**0.22**	0.09	0.31	-0.18
Chem35	alpha-Tochopherol_RT21.95	**0.50**	-0.07	0.01	-0.03	-0.14	-0.07	-0.16
Chem36	Cholesterol_RT21.95	-0.02	0.02	0.03	0.07	-0.03	-0.07	0.01
Chem37	Cholesta-5,7-dien-3-ol_RT22.27	0.16	0.00	0.00	-0.09	0.02	-0.10	-0.01
Chem39	Ergosterol (Ergosta-5,7,22-trien-3-ol)_RT22.79	0.12	0.02	0.01	0.06	-0.04	-0.03	-0.11
Chem40	Unidentified Steroid_RT22.95	-0.12	0.10	0.03	0.16	-0.11	-0.18	-0.07
Chem41	Campesterol (Ergost-5-en-3β-ol)_RT23.01	-0.14	0.07	0.07	**0.29**	-0.10	-0.02	-0.07
Chem42	Cholesta-4-en-3-one _RT23.26	-0.08	0.03	0.02	-0.02	0.02	**-0.26**	-0.05
Chem43	Ergosta-5,8-dien-3-ol_RT23.48	0.11	0.05	-0.02	0.11	0.07	0.04	-0.04
Chem44	Cholesta-4,6-dien-3-one_RT23.67	-0.03	0.06	-0.05	-0.07	0.16	0.05	**0.23**
Chem47	gamma-Sitosterol_RT24.00	-0.18	0.02	0.10	**0.42**	**-0.24**	0.01	0.03
Chem48	Stigmastanol_RT24.14	-0.12	0.03	-0.06	**0.23**	**-0.32**	**0.28**	**0.28**
Chem49	Unidentified Steroid_RT24.24	0.07	0.07	-0.10	-0.15	0.07	0.02	0.13
Chem51	Unidentified Steroid_RT24.48	0.13	0.07	-0.09	0.04	-0.15	0.10	-0.02
Chem57	Unidentified Waxy Ester_RT25.45	-0.03	0.10	0.05	0.01	0.10	-0.06	0.02
Chem58	Unidentified Waxy Ester_RT25.88	-0.04	**0.22**	**0.78**	**-0.34**	**-0.23**	**0.20**	0.14
Chem59	Unidentified Waxy Ester_RT26.14	**-0.32**	0.16	-0.11	**-0.36**	-0.10	0.19	**-0.64**
Chem62	Oleic acid, octadecyl ester _RT28.51	-0.16	0.13	-0.18	-0.08	0.07	0.08	0.03
Chem64	Hexadecanoic acid, eicosyl ester_RT28.72	-0.15	0.05	0.00	-0.02	0.17	**-0.23**	0.14
Chem66	Unidentified Waxy Ester _RT29.92	-0.09	**0.29**	**-0.37**	-0.14	**-0.48**	**-0.21**	0.03
	Standard Deviation	3.96	3.00	2.55	1.87	1.80	1.54	1.47
	Eigenvalue	15.66	9.01	6.48	3.51	3.24	2.37	2.16
	Variance	0.27	0.16	0.11	0.06	0.06	0.04	0.04
	Cumulative Variance	0.27	0.43	0.54	0.60	0.66	0.70	0.73

Table S4.4: Rotated factor loadings for PC1 to PC7 from within-lineage principal components analyses performed on the relative of the 42 compounds in the secretions of the experimental males. PC1 to PC7 were used for within-lineage analyses of chemical associations based on the enclosure experiment. Bold values indicate factor loadings considered strong (> |0.2|). Compounds in bold differed between the lineages in their relative abundance and those highlighted in grey were repeatable within individuals based on ICC values (see Table 4.1).

		ITA							WEUR						
ID	Compound	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Chem1	**Heptadecene_RT8.25**	-0.02	-0.14	-0.03	0.09	-0.18	**-0.21**	-0.01	0.14	0.00	-0.04	0.10	0.02	-0.07	0.02
Chem4	2-Heptadecanone_RT9.85	-0.05	-0.04	-0.02	0.12	-0.10	-0.16	-0.06	0.15	0.04	-0.06	0.05	0.02	-0.02	0.02
Chem7	Palmitic acid (Hexadecanoic acid)_RT10.3	**-0.43**	0.00	-0.11	0.09	-0.02	-0.04	0.09	**0.36**	**-0.34**	0.04	**-0.24**	0.00	**0.31**	**-0.27**
Chem9	2-Nonadecanone_RT11.5	-0.11	-0.04	-0.03	**0.31**	**-0.20**	-0.06	**0.53**	**0.20**	-0.02	-0.15	0.13	0.14	0.00	-0.19
Chem11	Oleic acid (9-Octadecenoic acid)_RT11.81	**-0.42**	**-0.26**	-0.02	-0.04	**0.26**	**0.29**	0.10	**0.50**	**-0.29**	0.00	-0.02	**0.21**	-0.11	0.02
Chem12	Stearic acid (Octadecanoic acid)_RT11.98	**-0.58**	-0.05	-0.17	-0.06	0.11	**-0.20**	**-0.22**	**0.30**	-0.14	-0.03	0.04	0.02	0.02	**0.23**
Chem15	Eicosanoic acid_RT13.70	0.00	-0.13	-0.18	0.05	**-0.29**	0.05	-0.08	0.06	**0.20**	0.18	**0.29**	-0.02	**0.24**	**0.43**
Chem16	Squalene_RT18.34	-0.04	0.04	-0.05	0.09	0.11	-0.14	**-0.27**	0.01	0.03	-0.05	-0.03	-0.15	-0.04	-0.11
Chem17	Unidentified_RT18.58	0.00	-0.13	0.14	0.01	-0.07	-0.01	0.05	0.03	0.04	0.16	**0.26**	-0.01	0.03	0.10
Chem18	Unidentified Steroid_RT19.1	0.00	0.01	0.15	0.06	0.05	-0.06	0.15	-0.02	0.00	0.02	-0.01	-0.04	**-0.17**	-0.03
Chem19	Cholesta-2,4,6-triene_RT19.22	-0.07	-0.01	0.18	0.02	-0.11	-0.12	-0.13	-0.05	0.05	-0.11	-0.12	**0.36**	-0.11	**0.32**
Chem20	Cholesta-3,5-diene_RT19.34	-0.02	-0.08	0.09	0.03	-0.12	-0.03	-0.04	0.09	0.05	-0.05	0.09	-0.06	-0.10	-0.09
Chem21	Unidentified Steroid_RT19.57	0.04	0.02	0.12	0.10	0.03	-0.05	-0.09	-0.09	-0.05	0.01	-0.10	-0.06	-0.11	-0.05
Chem23	**Unidentified Steroid_RT19.75**	0.00	0.00	0.18	0.08	0.07	0.02	0.02	-0.11	-0.05	0.05	-0.12	-0.04	-0.14	0.02
Chem24	Unidentified Steroid_RT19.86	0.06	0.02	0.08	0.09	-0.01	-0.01	-0.05	-0.08	-0.02	0.00	-0.03	-0.08	-0.11	-0.11
Chem25	Unidentified Steroid_RT19.97	0.02	0.02	**0.22**	0.05	0.08	-0.04	0.03	-0.14	-0.10	-0.02	-0.11	-0.06	-0.18	0.19
Chem26	**Unidentified Steroid_RT20.09**	0.03	0.04	0.06	0.11	**-0.30**	**0.32**	-0.01	-0.02	**0.33**	0.15	0.08	0.13	-0.04	**-0.31**
Chem27	Unidentified Steroid_RT20.24	0.08	0.09	0.08	-0.07	**0.21**	0.05	**0.30**	-0.11	0.07	0.38	-0.05	0.34	-0.09	**-0.20**
Chem28	Unidentified Steroid_RT20.40	-0.02	0.03	0.16	0.05	0.04	0.02	-0.02	-0.12	-0.04	-0.02	-0.18	-0.04	-0.16	0.12
Chem29	Unidentified Steroid_RT20.76	0.05	0.01	-0.01	0.06	0.01	-0.07	-0.06	-0.01	-0.01	0.01	-0.03	-0.08	-0.11	-0.07
Chem30	**Tocopherol methyl ether_RT20.78**	0.14	0.07	-0.14	0.15	0.19	-0.02	**-0.21**	0.02	-0.03	0.12	**-0.35**	-0.15	**0.31**	0.14
Chem31	Unidentified Steroid_RT20.99	0.02	0.12	0.02	-0.01	0.00	-0.04	0.14	-0.02	0.03	0.06	0.03	0.05	-0.12	-0.03
Chem32	**Unidentified Steroid_RT21.17**	0.05	**0.26**	0.05	-0.06	0.08	**0.37**	0.12	-0.16	0.05	**0.29**	0.01	0.19	0.07	-0.03
Chem35	**alpha-Tochopherol_RT21.95**	0.13	0.07	-0.01	0.10	0.18	0.10	**-0.35**	-0.03	-0.03	**0.20**	-0.14	-0.13	**0.28**	0.01
Chem36	**Cholesterol_RT21.95**	0.05	0.05	-0.07	0.02	-0.05	-0.01	-0.06	0.04	0.02	-0.02	0.01	-0.01	-0.01	-0.06
Chem37	**Cholesta-5,7-dien-3-ol_RT22.27**	0.04	0.04	0.03	0.08	0.00	0.02	-0.10	-0.03	0.01	-0.04	-0.06	-0.15	-0.09	-0.08
Chem39	**Ergosterol (Ergosta-5,7,22-trien-3-ol)_RT22.79**	0.07	0.09	-0.08	-0.08	-0.04	0.15	-0.02	-0.01	0.01	0.03	-0.02	-0.07	-0.06	-0.11
Chem40	**Unidentified Steroid_RT22.95**	0.11	0.08	**-0.22**	0.03	**-0.40**	0.09	0.09	-0.02	-0.03	**-0.15**	0.02	0.10	-0.04	0.02
Chem41	**Campesterol (Ergost-5-en-3β-ol)_RT23.01**	0.06	0.07	**-0.26**	-0.04	-0.07	0.04	-0.14	0.02	0.08	0.06	0.17	0.04	0.19	0.01
Chem42	Cholesta-4-en-3-one _RT23.26	0.04	0.08	0.02	-0.03	**-0.25**	-0.15	-0.11	0.08	-0.01	-0.13	0.11	**-0.22**	**-0.20**	0.01
Chem43	**Ergosta-5,8-dien-3-ol_RT23.48**	0.06	0.03	-0.03	0.01	0.00	0.04	-0.02	-0.10	0.03	**0.21**	0.06	-0.07	-0.02	-0.03
Chem44	Cholesta-4,6-dien-3-one_RT23.67	0.01	0.02	**0.19**	0.02	0.18	-0.12	**0.23**	-0.04	0.03	0.02	0.12	-0.10	-0.16	0.11
Chem47	**gamma-Sitosterol_RT24.00**	-0.01	0.08	**-0.46**	-0.10	0.05	-0.03	0.15	0.05	0.11	0.06	**0.22**	0.16	**0.26**	0.15
Chem48	**Stigmastanol_RT24.14**	0.01	0.24	**-0.32**	**-0.20**	**0.22**	**0.22**	0.07	-0.01	-0.04	-0.04	**-0.24**	0.14	-0.16	**0.35**
Chem49	Unidentified Steroid_RT24.24	0.05	-0.01	**0.16**	0.05	0.04	-0.08	-0.06	-0.14	-0.10	0.01	-0.14	-0.06	-0.17	0.05
Chem51	**Unidentified Steroid_RT24.48**	0.07	0.07	-0.06	-0.05	0.00	0.03	-0.01	-0.17	-0.11	0.09	**-0.20**	0.04	**0.21**	-0.15

Chem57	Unidentified Waxy Ester_RT25.45	0.13	-0.04	0.03	0.10	0.05	0.07	-0.11	-0.03	0.05	-0.04	0.10	0.05	-0.05	-0.18
Chem58	Unidentified Waxy Ester_RT25.88	**0.37**	**-0.72**	**-0.24**	-0.07	**0.23**	-0.09	0.09	0.18	**0.68**	**-0.35**	**-0.37**	-0.13	0.16	-0.02
Chem59	**Unidentified Waxy Ester_RT26.14**	-0.07	**-0.22**	**0.23**	**-0.71**	**-0.28**	0.16	-0.09	-0.12	-0.04	**-0.47**	**0.23**	**0.27**	0.06	-0.16
Chem62	**Oleic acid, octadecyl ester _RT28.51**	-0.05	0.04	**0.21**	-0.05	0.15	0.13	-0.13	**-0.22**	-0.06	-0.05	0.08	-0.04	0.13	-0.10
Chem64	**Hexadecanoic acid, eicosyl ester_RT28.72**	0.07	-0.10	0.09	-0.01	0.08	0.11	0.11	0.04	-0.11	0.02	0.29	**-0.55**	0.02	0.00
Chem66	Unidentified Waxy Ester _RT29.92	0.11	**0.29**	0.00	**-0.42**	0.07	**-0.55**	0.16	**-0.40**	**-0.27**	**-0.36**	0.06	0.04	**0.37**	0.07
	Standard Deviation	3.40	2.44	2.37	2.12	1.79	1.65	1.43	3.19	2.65	2.33	2.06	1.69	1.58	1.44
	Eigenvalue	11.56	5.93	5.61	4.48	3.20	2.71	2.05	10.16	7.00	5.43	4.26	2.87	2.48	2.07
	Variance	0.25	0.13	0.12	0.10	0.07	0.06	0.04	0.22	0.15	0.12	0.09	0.06	0.05	0.04
	Cumulative Variance	0.25	0.38	0.50	0.60	0.67	0.73	0.77	0.22	0.37	0.49	0.59	0.65	0.70	0.75

Table S4.5: Rotated factor loadings for PC1 (Body Size) from a principal components analyses performed on three body size related traits: snout-vent length (SVL), head length and mass.

Trait	Body Size (PC)
SVL	0.93
Head Length	0.25
Mass	0.26
Standard Deviation	4.32
Eigenvalue	18.66
Proportion of Variance	0.96

Table S4.6: Rotated factor loadings for PC1 to PC6 from a principal components analyses performed on the relative abundance of 26 compounds in the secretions of males from the secondary contact zone. PC1 to PC6 were used to generate a chemical index for the analysis of patterns of chemical introgression. Bold values indicate loadings considered strong (> |0.20|). Compounds in bold differed in relative abundance between the lineages and those highlighted were repeatable within individuals based on ICC values (see Table 4.1).

Compound ID	PC1	PC2	PC3	PC4	PC5	PC6
Heptadecene_RT8.25	0.14	0.00	0.15	**-0.36**	**0.26**	-0.02
2-Heptadecanone_RT9.85	0.09	-0.09	0.10	-0.19	0.15	-0.04
Squalene_RT18.34	-0.10	-0.20	-0.07	0.15	-0.02	**-0.40**
Unidentified Steroid_RT19.1	0.01	-0.16	-0.07	0.04	0.12	-0.03
Cholesta-3,5-diene_RT19.34	-0.04	-0.03	0.01	-0.06	0.13	-0.08
Unidentified Steroid_RT19.57	-0.04	-0.18	-0.09	0.08	0.11	-0.01
Unidentified Steroid_RT19.75	-0.02	**-0.25**	-0.12	0.16	0.11	-0.01
Unidentified Steroid_RT19.97	-0.02	-0.16	-0.11	0.12	0.14	-0.05
Unidentified Steroid_RT20.4	-0.02	**-0.20**	-0.11	0.15	0.10	-0.10
Unidentified Steroid_RT20.76	0.04	-0.04	-0.06	0.07	-0.01	0.05
Tocopherol methyl ether_RT20.78	**-0.38**	0.12	0.16	**-0.28**	-0.06	-0.16
Unidentified Steroid_RT21.17	-0.07	0.00	-0.03	-0.09	0.05	-0.16
alpha-Tochopherol_RT21.95	**-0.66**	0.15	0.14	**-0.24**	-0.19	0.03
Cholesterol_RT21.95	0.03	**0.49**	**0.58**	**0.53**	**0.29**	-0.04
Cholesta-5,7-dien-3-ol_RT22.27	-0.12	-0.14	0.00	0.08	0.01	0.04
Ergosterol (Ergosta-5,7,22-trien-3-ol)_RT22.79	-0.03	-0.04	-0.06	0.13	**-0.20**	**0.57**
Unidentified Steroid_RT22.95	0.18	0.02	0.05	0.04	-0.14	-0.14
Campesterol (Ergost-5-en-3β-ol)_RT23.01	**0.26**	0.12	0.07	-0.07	**-0.22**	0.18
Cholesta-4-en-3-one _RT23.26	**0.26**	0.04	0.11	**-0.47**	**0.30**	**0.29**
Ergosta-5,8-dien-3-ol_RT23.48	-0.10	-0.02	0.00	0.15	-0.12	**0.36**
Cholesta-4,6-dien-3-one_RT23.67	0.15	-0.02	-0.06	0.05	-0.01	0.03
gamma-Sitosterol_RT24.00	**0.38**	0.06	0.12	-0.13	**-0.45**	**-0.38**
Stigmastanol_RT24.14	0.06	0.03	0.03	0.03	**-0.45**	0.04
Unidentified Steroid_RT24.24	-0.04	-0.11	-0.04	0.04	0.17	0.07
Unidentified Steroid_RT24.48	0.00	-0.06	-0.01	0.09	-0.18	0.05
Unidentified Waxy Ester_RT25.45	0.01	**0.66**	**-0.69**	0.00	0.11	-0.08
Standard Deviation	2.39	1.85	1.82	1.62	1.29	1.07
Eigenvalue	5.70	3.43	3.32	2.64	1.67	1.15
Proportion of Variance	0.24	0.15	0.14	0.11	0.07	0.05
Cumulative Proportion of Variance	0.24	0.39	0.53	0.64	0.71	0.76

Table S4.7: The relative abundance of chemical compounds in male secretions presented by chemical class and lineage. Values represent the average relative abundance and 95% confidence interval ($CI_{95\%}$) from the sum, for each male, of the transformed relative abundances of compounds within each chemical class. Bold values indicate statistical difference between the lineages.

Chemical Class	Compounds	ITA	WEUR
Alkene	1	**-0.03 (-0.32,0.25)**	**0.65 (0.43,0.87)**
Ketone	2	-2.19 (-2.74,-1.65)	-1.65 (-2.23,-1.07)
Carboxylic acid	4	2.16 (0.27,4.06)	3.59 (2.00,5.17)
Terpenoid	1	0.97 (0.64,1.30)	0.60 (0.44,0.76)
Steroid	25	-2.36 (-4.14,-0.57)	-3.17 (-5.10,-1.25)
Tocopherol	2	**4.34 (3.69,4.99)**	**-3.07 (-3.75,-2.38)**
Waxy Ester	6	**-1.30 (-2.71,0.12)**	**4.73 (3.31,6.15)**
Unknown	1	-0.99 (-1.25,-0.74)	-0.89 (-1.02,-0.76)

Table S4.8: Within-lineage Pearson's correlation coefficients between male traits and the chemical profiles (defined as PC1 to PC7, see Table S4). All traits were standardized (mean = 0, SD = 1) prior to analysis.

	ITA							WEUR						
	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Dominance	-0.05	0.18	0.23	0.01	0.41	-0.07	-0.02	0.18	0.03	0.10	0.09	0.01	-0.47	-0.09
Body Size	-0.50	0.01	-0.02	-0.19	-0.08	-0.11	0.04	0.08	-0.11	0.16	-0.04	-0.31	0.05	0.15
Bite Force	-0.26	0.17	0.24	-0.18	0.20	-0.03	-0.13	-0.19	-0.24	0.15	-0.08	-0.01	-0.09	0.10
Testes Mass	-0.27	0.29	0.03	-0.28	-0.08	-0.19	-0.19	-0.24	-0.31	-0.10	-0.05	0.00	-0.19	0.31
Greenness	-0.06	0.17	-0.11	0.10	0.13	-0.20	0.32	-0.02	-0.26	0.04	0.06	0.11	-0.26	-0.11
Blackness	0.03	0.02	-0.27	-0.15	0.25	-0.03	0.18	-0.31	0.10	0.01	0.07	-0.24	-0.45	0.00
Blue Area	-0.12	-0.11	0.04	-0.16	0.29	0.06	0.22	-0.02	-0.16	0.09	0.03	0.20	0.12	0.31
OVS Hue	-0.23	0.01	-0.33	-0.14	-0.14	0.27	0.01	0.04	0.30	-0.04	-0.11	-0.24	0.00	0.05
OVS UV Chroma	0.14	-0.01	-0.11	0.23	0.31	0.29	-0.16	0.18	0.07	0.31	-0.19	0.28	-0.20	0.15

Table S4.9: Results from MANOVA's performed within-lineage for body size and performance traits (body size, bite force and testes mass) and colouration (greenness, blackness, OVS blue area, OVS hue and OVS UV Chroma) as multivariate responses and PC1-PC7 (Table S4) as putative chemical predictors.

		ITA			WEUR		
Multivariate Response	Chemical Component	Pillai	F (approximated)	p	Pillai	F (approximated)	p
Body Size and Performance	PC1	0.28	2.52	0.09	0.12	0.87	0.47
	PC2	0.15	1.11	0.37	0.14	1.12	0.36
	PC3	0.09	0.61	0.62	0.08	0.61	0.62
	PC4	0.12	0.84	0.49	0.03	0.22	0.88
	PC5	0.03	0.17	0.92	0.18	1.48	0.25
	PC6	0.09	0.66	0.59	0.07	0.47	0.71
	PC7	0.07	0.49	0.69	0.12	0.88	0.47
Colouration	PC1	0.16	0.71	0.63	0.20	0.59	0.71
	PC2	0.18	0.77	0.59	0.30	1.05	0.43
	PC3	0.37	2.14	0.11	0.41	1.68	0.21
	PC4	0.15	0.64	0.67	0.30	1.01	0.45
	PC5	0.38	2.18	0.10	0.26	0.86	0.53
	PC6	**0.50**	**3.54**	**0.02**	**0.61**	**3.69**	**0.03**
	PC7	0.11	0.46	0.80	0.28	0.91	0.50

Table S4.10: AICc for genetic and phenotypic cline models (Hybrid Index: Model I - none, fixed, Model II – left, fixed, Model III – right, fixed, Model IV – mirror, fixed, Model V – both, fixed, Model VI – none, free, Model VII – left, free, Model VIII – right, free, Model IX – mirror, free, Model X, both, free; Phenotypic Characters: Model I - none, Model II – left, Model III – right, Model IV – mirror, Model V – both).

Model	Model I	Model II	Model III	Model IV	Model V	Model VI	Model VII	Model VIII	Model XI	Model X
Hybrid Index	19.7	23.6	**13.4**	19.1	17.1	22.3	26	16.4	22.9	20.6
Chemical Index	-155.6	-151.9	-154.2	**-156.8**	-149					
Greenness	**512.1**	512.3	516.2	512.5	514					
Tocopherol methyl ether	**-45.3**	-43.9	-41.3	-44.4	-39.5					
alpha-Tocopherol	**-72.1**	-67.4	-67.4	-67.4	-63.6					

Chapter 5

Comparison of reproductive effort in native and non-native populations reveals sex differences in adaptive potential

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5.1 Abstract

Non-native animals can encounter very different environments than those they are adapted to. Functional changes in morphology, physiology and life-history following introduction show that organisms can adapt both fast and efficiently. It remains unclear, however, if female reproductive characters and male sexually selected behaviour show the same adaptive potential. The common wall lizard, *Podarcis muralis*, has been repeatedly introduced from Southern Europe to England over the past 80 years. Lizards in England experience a cool, seasonal climate that effectively restricts recruitment to the first clutch of the season, whereas in their native range up to three clutches per season recruit. As a consequence, both females and males in non-native populations should benefit from reducing or even eliminating their reproductive investment in second clutches. Using a combination of field data and experiments, we show that non-native females produce relatively larger and heavier first seasonal clutches and smaller and lighter second seasonal clutches compared to native females. In contrast, non-native and native males do not differ in their territorial and sexual behaviour later in the season. An adaptive shift in male seasonal reproductive investment may be constrained because males use breeding females as cues for sexual behaviour. If this is so, we expect a general pattern across climatic regimes whereby female reproductive investment evolves first, with responses in males lagging behind.

Keywords: Climate, Life-history, Behaviour, Sexual Selection

5.2 Introduction

Introduced species are outstanding models to study phenotypic evolution (Reznick & Ghalambor 2001; Prentis *et al.* 2008). New abiotic and biotic conditions can abruptly change selective regimes and cause rapid shifts in morphology, behaviour, physiology and life-history (e.g. Blossey & Notzold 1995; Huey *et al.* 2000; Yeh & Price 2004). Phenotypic variation along environmental clines typically involves both plasticity and genetic divergence, and the same is true for differences between native and non-native populations Although it is often difficult to know if the observed shifts in non-native populations are in the direction favoured by selection, this inference is strengthened when the adaptive value of phenotypic clines in the ancestral range is well established (e.g. Gilchrist *et al.* 2001; Hoffmann *et al.* 2002).

Reproductive life-history traits (e.g. relative investment, timing, frequency and duration of reproductive events) often vary adaptively within and between species with changes in temperature and seasonality, along latitudinal and altitudinal gradients (e.g. Niewiarowski 1994; Rose & Lyon 2013; Du *et al.* 2014). For example, in multi-clutching ectotherms, females in populations at high latitudes typically invest relatively more in the first seasonal reproductive event (e.g. Forsman & Shine 1995; Roig *et al.* 2000), sometimes resulting in the production of a single clutch per year in a cool climate and several clutches in a warm climate (Pincheira-Donoso & Hunt 2015). We therefore predict that females introduced to a comparably cooler climate should exhibit a similar seasonal shift in reproductive allocation.

Climatic effects on the timing, frequency, duration or success of female reproductive events should also cause concomitant variation in the adaptive value of male investment in reproduction across the breeding season. Thus, males should modify their sexual behaviour in accordance with expected fitness returns on investment (Hirshfield & Tinkle 1975). Indeed, numerous experimental studies have shown that males adjust their competitive behaviour and

courtship effort based on the prevailing reproductive environment (e.g. Grant *et al.* 1995, Svensson *et al.* 2010), including in response to female reproductive potential (e.g. Reading & Backwell 2007). Furthermore, sexual selection regimes can also change in response to changes in mating opportunities (Shuster & Wade 2003), with potential implications for the relative costs and benefits of male sexual morphology. For example, variance in the ratio of receptive females to males (the operational sex ratio) between reproductive episodes could generate seasonal variation in the intensity and direction of sexual selection (e.g. Reichard *et al.* 2008; Wacker *et al.* 2014). If females become more synchronous in their receptivity, as is predicted in more seasonal environments (e.g. Ramírez-Pinilla *et al.* 2009), dominant males will be less able to monopolise multiple females, reducing the opportunity for sexual selection (e.g. Grant *et al.* 1995, Mendoza-Cuenca & Macias-Ordonez 2009). Thus, in response to a new climatic regime, the expression of male sexually selected morphology could also shift in non-native populations, however the direction of change, if any, is not easy to predict.

These considerations suggest that female reproductive life-history and male reproductive behaviour should shift concurrently following introduction to a different climatic regime which could result in changes to sexually selected traits. However, there is limited evidence that this is the case, and it is possible that female and male reproductive characters do not have similar adaptive potential. Here we take advantage of a series of introductions of common wall lizards, *Podarcis muralis* (Laurenti, 1768), from Italy into England, where the spring and summer temperatures are substantially lower. Extant populations in England are well characterized genetically and most originate from north-central Italy (approximately Tuscany and Bologna-Modena, Michaelides *et al.* 2015). In Italy, where the species is native, females have up to three clutches per breeding season. However, climatic conditions in England place significant restrictions on embryo development, resulting in highly reduced recruitment from second clutches (While *et al.* 2015). Thus, female and male fitness is almost entirely dependent on their reproductive success in the first clutch of the season, with second clutches contributing little to

the total number of surviving offspring produced. As a consequence, in non-native females we expect investment in second clutches to be reduced in favour of first clutches relative to native females. Further, we expect non-native males to invest less in their reproductive behaviour towards second clutches compared to native males. If reproductive responses in females have consequences for sexual selection regimes, this could also result in adaptive divergence between native and non-native populations in male sexually selected traits. We tested these predictions using a combination of field data and experiments in outdoor enclosures. Specifically, we (i) tested for divergence in female reproductive investment and male sexual characters (e.g. body size, head size, bite force, colouration), and the degree and direction of sexual dimorphism between native and non-native populations, (ii) examined differences in patterns of reproductive investment in first and second clutches between females from the native and non-native range under standardized conditions, and (iii) explored in experimental populations whether any shifts in female reproductive investment were accompanied by differences between native and non-native males in the intensity of male-male competition and courtship effort for second clutches.

5.3 Materials and Methods

Study populations

The common wall lizard, *P. muralis*, is a small diurnal lacertid native to southern Europe. The species has established non-native populations within Europe and North America over the last century, primarily through the pet trade and deliberate introductions (Deichsel & Gist 2001; Schulte 2008; Schulte *et al.* 2012b; Michaelides *et al.* 2015; While *et al.* 2015). From 2010 to 2015 we captured 478 native (females (n = 196), males (n = 282)) and 655 non-native (females (n = 372), males (n = 283)) adult lizards (≥45mm Snout-vent length (SVL)) from ten non-native populations in the south of England (Figure 5.1) and eighteen native populations in northern Italy (Table S5.1). The first recordings of individuals at the non-native localities ranges from 1930 to 2004 and the native sources were identified genetically in a previous study (Michaelides *et al.* 2015). All populations (native and non-native) included in our analyses are of pure Italian (Tuscan and/or Venetian ancestry) and have a green-backed morphology.

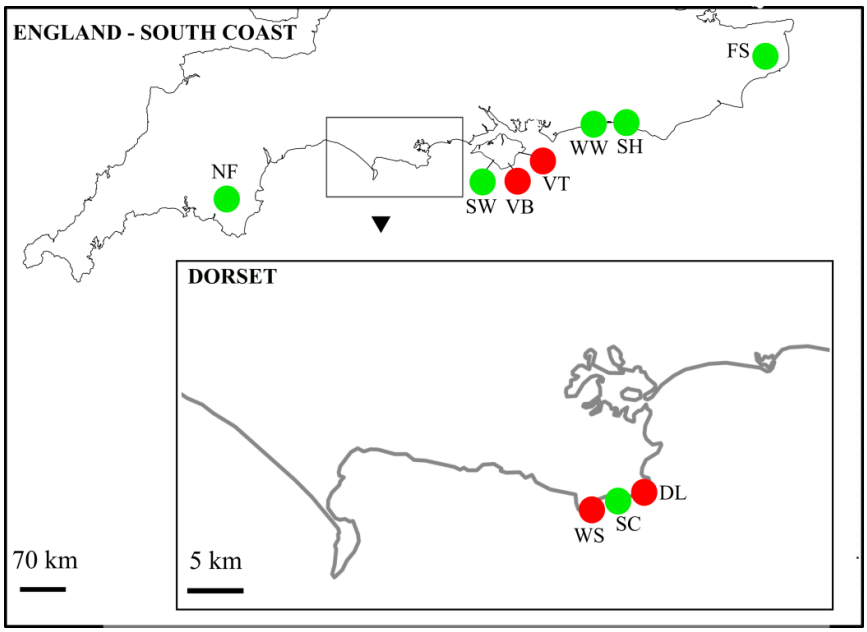

Figure 5.1: Map to show the locations of non-native wall lizard populations sampled for this study. Individuals from populations in red were used in the enclosure experiment (see below).

Morphological divergence

We captured all lizards at the start of the breeding season (March-April) to ensure that they were within their first seasonal reproductive episode. Abdominal palpation confirmed the presence of eggs in all females in this study unless otherwise stated. Upon capture, we recorded four morphometric measurements from each lizard: SVL, measured with a ruler to the nearest mm, Body Mass, measured to the nearest 0.01 g using digital scales, and Head Length and Head Width, recorded to the nearest 0.1 mm with callipers. In addition, two authors (GMW and TU) scored dorsal green colouration (Greenness), based on an intensity scale from 1 to 10. One author (GMW) photographed all individuals on their ventral and left lateral side against an X-rite Colour-Checker chart using a Canon EOS 350D digital camera with white balance customised prior to each photo session to adjust for the background illumination (e.g. Wang & Shaffer 2008). From these photographs, we estimated ventral blackness (Blackness) from the chest section (the region from the collar to the forelimbs) and outer ventral scale blue spot area (OVS Blue Area) from lateral images using the program ImageJ (available at http://imagej.nih.gov). For a sample of native and non-native lizards, which we returned to laboratory facilities at Oxford University in 2013 and 2014, we also recorded maximum bite force (Bite Force, n = 122), and quantified outer ventral scale UV chroma (OVS UV Chroma, n = 94) and hue (OVS Hue, n = 94) from reflectance spectra of males. Expanded details on the quantification of traits are given in the Supplementary Information, and Table S5.2 gives all sample sizes by trait, origin and sex.

Female reproductive investment

We returned a sample of females (n = 303) collected between 2010 and 2015, which were carrying their first clutch of the season, to the laboratory. We housed the females individually within plastic terraria (590 × 390 × 415 mm) that contained sand substrate, a basking block, a shelter, and moist sand for egg laying. We kept the females under a 12:12 light/dark cycle. A 60

Watt spotlight above each cage provided opportunities for thermoregulation and UV light was provided with EXO-TERRAT 10.0 UVB fluorescent tubes. We fed the lizards daily (either two mealworms or two crickets) and sprayed the cages with water every second day. During this time, we checked the cages at least twice daily (am and pm) for eggs. To quantify female reproductive investment, we retrieved and counted the number of eggs within each clutch, and noted the presence and number of infertile eggs (following Olsson & Shine 1997a). In addition, we measured clutch mass (fertile eggs only) and post-parturition body mass using a digital balance that recorded to the nearest 0.01 g. We housed 72 females collected in 2014 (native (n = 40), non-native (n = 32)) in experimental enclosures (see below) during their second seasonal receptive phase.

Male reproductive investment and sexual selection

Outdoor enclosure experiment

Lizards captured in 2014 from five native populations (80 lizards) and four non-native populations (64 lizards) were used in an enclosure experiment to compare reproductive investment towards second clutches in native and non-native lizards (see Table S5.1 for population details). The native and non-native localities differ substantially in their thermal environment with mean monthly maximum air temperatures during the main activity season for the populations in England approximately 5–10 °C lower than their source regions in northern Italy (see While et al. 2015). The genetic origin of the four non-native populations can be traced to at least three sources in the native range (Michaelides *et al.* 2015). We obtained tissue samples from all individuals for genetic analyses by removing the tip of the tail, and preserved these in 90% ethanol. We confirmed the presence of eggs by palpating each female. In five cases the female had recently laid their first seasonal clutch prior to capture. We kept these females cool (4 °C) to delay ovulation. All other lizards were housed individually as above until they oviposited. We kept most females for two days under lab conditions post-oviposition before their inclusion in the enclosure experiment. However, to enable the simultaneous release

of receptive females into the enclosures, we kept some females at 4 °C for additional days following oviposition to avoid progression through the next ovulation cycle.

We assigned each lizard to one of nine (~ 7 × 7m) outdoor enclosures at the John Krebs Field Station, University of Oxford, where the climate falls within the variation in the non-native range of wall lizards in England (While *et al.* 2015; Thermocron temperature loggers recorded mean daily temperatures in our enclosures that ranged from 11.6 to 22.6 °C during the experiment). Each enclosure housed sixteen lizards of either native or non-native origin (8 male, 8 female). This is within the range of densities found under natural conditions (While & Uller, personal observations). For all enclosures individuals came from at least four populations with a minimum of three populations represented within each sex (see SI for further details on assignment). Prior to release, we marked all lizards for identification at a distance with a unique number on their dorsal side using a non-toxic, non-hypoallergenic marker pen (Mitsubishi Pencil Company Ltd). We released males a minimum of six days prior to the release of females to enable them to establish territories. Lizards of each sex were released within an enclosure simultaneously except in three cases where a single female was released within three days of the initial release of all the other females. At completion of the experiment, we recaptured and returned the lizards to the laboratory and housed them under standardized conditions (see above). Five males (two native and three non-native) and one female (native origin) were not recaptured and presumed dead. Four non-native females did not produce a second seasonal clutch of eggs. For the remaining females (native (n = 39) and non-native (n = 28)) we recorded investment in second clutches, and took tail tissue samples from all second clutch juveniles to be used for the assignment of offspring paternity.

Collection of behavioural data

To quantify male investment in territory establishment and courtship, one author (HEAM) systematically observed the enclosures from the initial release of males until we confirmed that

the females were in the late stages of gestation. During this time (~4 weeks) the observer recorded behavioural interactions within each enclosure during 45 minute observation periods. The identity of interacting lizards, the initial location of the receiver, and the nature of the social interaction were recorded according to an ethogram following Heathcote *et al.* (2016). From the observations, we categorised male-male territorial interactions (n = 414, the identity of both males was known in 395) and courtships (n = 511, mating was observed in 92) (see SI for further details). For each male we quantified: total number of competitive interactions; relative number of courtships; relative number of females courted; relative mating success and relative fertilization success. The latter four measures were relative to the average of all males of the same enclosure and mating and fertilization success were based on paternity data (see below).

We calculated Dij-based David's Dominance scores for each male within an enclosure (hereafter Dominance) based on the outcome of observed dyadic male-male territorial interactions. Dominance was calculated in R package 'Steepness' (de Vries 2011) following Gammell *et al.* (2003) with correction (to control for differences in the numbers of interactions between dyads) and normalisation (to control for the loss of males from three enclosures) described by de Vries et al. (2006).

Paternity assignment

Following the experiment, native females produced 211 offspring (from 39 females in five enclosures), and non-native females produced 145 offspring (from 27 females in four enclosures) from their second clutches. We isolated DNA from offspring and adult tissue samples following QIAGEN DNeasy extraction protocol (Qiagen, Shanghai, China) in a final elution volume of 150 µl (in AE buffer). We carried out PCR reactions for 16 microsatellite markers with primers combined into five multiplexes (Table S6 Richard *et al.* 2012; Heathcote *et al.* 2015), and assigned paternity using Cervus version 3.0 (Marshall *et al.* 1998), based on the trio (mother, father, offspring) LOD score using a strict confidence level of 95%. Offspring with

more than one mismatching allele (21 native offspring) among mother-offspring-father trios and that amplified at fewer than three loci (one native offspring) were excluded from further analyses.

Strength and opportunity for sexual selection on males

To characterize and compare sexual selection on native and non-native males during the second within-season reproductive episode, we used a multiple index approach based on variance in mating and fertilization success (Jones 2009; Henshaw *et al.* 2016). For males of each origin, we estimated: (1) the Bateman gradient (βss), the slope of the least squares regression of relative mating success on relative fertilization (Jones 2009), (2) opportunity for overall selection (I), the variance in absolute fertilization success over the square of the mean fertilization success (Crow 1958), (3) the opportunity for sexual selection (I_S), the variance in absolute mating partners over the square of the mean total mating partners (Wade & Arnold 1980), and (4) the maximum standardized selection differential or Jones Index (S'_{max}), the product of β_{ss} and the square root of I_s (Jones 2009).

Statistical Analyses

All statistical analyses were conducted in R version 3.1.2 (R Core Team 2014, http://www.R-project.org/) unless otherwise stated. For linear mixed models (LMMs) and generalised linear mixed models (GLMMs) the significance of fixed effects are reported based on Type III F-tests (with Kenward-Roger's approximation) and likelihood-ratio tests, respectively. All mixed model analyses of female investment included Population nested within Origin as a random effect. Enclosure was included as a random effect in mixed model analyses of male behaviour and sexual selection. For models with a significant interaction term including Origin (native or non-native), we performed post-hoc tests to identify the sources of variation (implemented in R

package multcomp, Hothorn *et al.* 2008), and report p-values that are adjusted for multiple comparisons.

Tests for divergence in morphology

To test for divergence in morphology and sexual dimorphism between native and non-native lizards we ran LMMs with each morphological trait as a response variable and Sex, Origin and a Sex by Origin interaction as fixed effects, and SVL as a covariate (where applicable). Because Head Length and Head Width were highly correlated (r = 0.81), we excluded Head Width from analyses of divergence.

Tests for differences in female investment

We tested for differences in female reproductive investment (Clutch Size, Clutch Mass and Mean Egg Mass) in first clutches with a Poisson GLMM (for Clutch Size) and LMMs (for Clutch Mass and Mean Egg Mass) taking Origin, Post-Parturition Body Mass and Year as fixed effects. To test for differences between native and non-native experimental females in their reproductive allocation between first and second clutches we ran LMMs for all three measures of investment with Origin, Clutch for female (first or second), their interaction, and Post-parturition Body Mass as fixed effects, and Female ID nested within Population and Origin as a random effect.

Tests for differences in male sexual behaviour

We carried out a series of statistical tests to examine differences between the native and non-native experimental populations in male sexual behaviour and the intensity of male-male competition. First, because the steepness of linear dominance hierarchies may reflect the intensity of competition among males (Flack & de Waal 2004), we tested for differences in the dominance hierarchies of native and non-native males using a LMM with Dominance as the

response variable, and Origin, Dominance Rank (where 1 is the most dominant and 8 the least dominant male within an enclosure), and their interaction as fixed effects. To examine whether associations between territoriality and sexual behaviour differed between native and non-native males, we performed LMMs with relative number of courtships, relative number of females courted, and relative mating success as response variables. All models included Dominance (standardized: mean = 0, SD = 1) as a fixed effect and SVL (standardized: mean = 0, SD = 1) as a covariate.

Since body size is thought to be under strong sexual selection in wall lizards (Sacchi *et al.* 2009), and we predicted a relaxation in sexual behaviour for non-native males, we tested for Origin differences in the extent to which body size predicted male territorial and sexual behaviour. To generate an overall measure of male body size, we collapsed SVL, Head Length, Head Width and Body Mass into a single principle component (Body Size, Table S5.3). For number of competitive interactions, we performed a Poisson GLMM with Body Size, Origin and their interaction as fixed effects. For relative number of courtships, relative number of females courted, and relative mating success we performed LMMs with Body Size (standardized: mean = 0, SD = 1), Origin and their interaction as fixed effects.

We used Mantel permutation tests (10,000 iterations) implemented in SocProg 2.4 (Whitehead 2009) to establish whether male investment in courting females predicted patterns of paternity (see below) i.e. as a possible indicator of post-copulatory processes (Olsson & Madsen 1998b). Tie strengths for each male-female dyad were defined as absolute number of courtships and total number of offspring sired, for courtship and genetic networks, respectively. The p-values for native and non-native enclosures were combined using Fisher's method (Fisher 1932).

Comparison of the strength and opportunity for sexual selection

To compare the Bateman gradients of native and non-native males we ran a LMM with relative fertilization success as the response variable taking relative mating success, Origin and an interaction between relative mating success and Origin as fixed effects. To compare the opportunities for selection on native and non-native males we performed a Levene's Test (Levene 1960) and a Modified Levene's Test (Brown & Forsythe 1974) on variance in male mating success (normally distributed) and variance in male fertilization success (non-normally distributed), respectively.

We tested for differences between native and non-native males in the associations between sexual traits and relative fertilization success since this could indicate shifts in male mating effort. Each trait was included as a fixed effect in a model with male Origin and a trait by Origin interaction, and relative fertilization success as the response variable. Traits were standardized (mean = 0, SD = 1) prior to analysis and SVL was included as a covariate where appropriate.

To compare the levels of multiple paternity in the native and non-native enclosure populations we ran a Poisson GLMM with number of fathers per clutch as the response variable, Origin as a fixed effect, and Clutch Size as a covariate.

5.4 Results

Morphological divergence

Snout-vent length was less sexually dimorphic in non-native populations (Table 5.1), which was due to larger non-native females compared to native females ($p < 0.001$). In contrast, Head Length, Greenness, Blackness, OVS Blue Area and Bite Force were more sexually dimorphic in non-native populations (Table 5.1). This was also largely driven by shifts in female traits as opposed to male traits. Specifically, with the exception of Blackness ($p = 0.86$), non-native females had a significant reduction in each of these traits compared to native females after accounting for SVL ($p < 0.05$), whereas there were no significant differences between native and non-native males ($p > 0.05$). Furthermore, outer ventral scale ornamentation showed no significant divergence between native and non-native males (Table 5.1, Figure S5.2).

Table 5.1: Summary statistics from LMMs testing for differences in the degree of sexual dimorphism between native and non-native lizards. For OVS UV Chroma and OVS Hue, where only male data are available, tests for divergence between the two origins are reported. All models included population nested within origin as a random effect and SVL as a covariate. Significant effects are highlighted in bold based on a threshold of $\alpha < 0.006$, adjusted from the nominal $\alpha < 0.05$ following Bonferroni correction for the number of tests performed on these data. SVL, Greenness, OVS Blue Area, Bite Force, and OVS Hue were transformed (square root) prior to analysis.

	Origin	Sex	Origin x Sex	SVL
SVL (mm)	**$F_{1,24}$ = 13.36, p = 0.001**	**$F_{1,1121}$ =26.19, p < 0.001**	**$F_{1,1121}$ = 14.66, p < 0.001**	
Body Mass (g)	$F_{1,24}$ = 0.20, p = 0.65	**$F_{1,819}$ =137.72, p < 0.001**	$F_{1,819}$ = 4.04, p = 0.045	**$F_{1,824}$ = 2268.25, p < 0.001**
Head Length (mm)	$F_{1,23}$ = 5.94, p = 0.01	**$F_{1,1107}$ =1875.67, p < 0.001**	**$F_{1,1108}$ = 17.19, p <0.001**	**$F_{1,1108}$ = 1015.09, p < 0.001**
Greenness	$F_{1,25}$ = 2.51, p = 0.13	**$F_{1,1088}$ =101.55, p < 0.001**	**$F_{1,1089}$ = 19.65, p < 0.001**	**$F_{1,1097}$ = 310.37, p < 0.001**
Blackness (%)	$F_{1,14}$ = 0.02, p = 0.88	**$F_{1,562}$ =311.14, p < 0.001**	$F_{1,562}$ = 5.88, p = 0.02	**$F_{1,569}$ = 50.21, p < 0.001**
OVS Blue Area (mm^2)	$F_{1,12}$ = 8.30, p = 0.014	**$F_{1,487}$ = 458.95, p < 0.001**	**$F_{1,487}$ = 20.42, p < 0.001**	**$F_{1,487}$ = 28.25, p < 0.001**
Bite Force (N)*	$F_{1,9}$= 1.24, p = 0.29	**$F_{1,201}$= 442.59, p < 0.001**	**$F_{1,202}$ = 9.09, p = 0.003**	**$F_{1,203}$ = 177.41, p < 0.001**
OVS UV Chroma	$F_{1,8}$ = 0.21, p = 0.66			$F_{1,89}$ = 0.07, p = 0.79
OVS Hue (nm)	$F_{1,8}$ = 0.20, p = 0.67			$F_{1,88}$ = 2.20, p = 0.14

* For analysis of Bite Force, we controlled for body temperature at testing ($F_{1,205}$ = 1.43, p = 0.23)

Female reproductive investment

After accounting for differences in SVL, native females were heavier on average post-parturition of their first clutches than non-native females (least-squares means: native: 4.98 ± 0.15, non-native: 4.51 ± 0.12; $F_{1,16} = 5.64$, $p = 0.03$). Across all years and populations, non-native females produced larger and heavier first clutches than native females relative to post-parturition body mass (Figure 5.2, Table S5.6). Year of collection also explained significant variance in clutch mass and mean egg mass but not in clutch size (Table S5.6). The probability of producing a second clutch was significantly lower for non-native females compared to native females (100 % of native females (39/39) and 88% of non-native females (28/32) produced a second clutch, $\chi^2 = 5.17$, $p = 0.01$). For females that produced both a first clutch in the wild and a second clutch in our enclosures, the duration (days) between oviposition of first and second clutches did not differ significantly between native and non-native females (native: 37.4 ± 0.7 and non-native: 38.6 ± 1.3 days, Origin: $\chi^2 = 0.58$, $p = 0.45$). Infertilities occurred within five first clutches (3 native and 2 non-native) and eight second clutches (1 native and 7 non-native) but in only one instance (non-native) was a female's entire clutch infertile. Analyses of relative female investment in first and second clutches showed a significant interaction effect between Origin and Clutch (first or second) for all three measures of investment (Table 5.2, Figure 5.3, also see Table S5.7 for results from models excluding post-parturition body mass and including SVL). Post-hoc tests revealed that the significant sources of variation were larger first clutch size ($p = 0.05$), and heavier first clutch mass ($p = 0.03$) in non-native compared to native populations, and heavier clutch mass in non-native first clutches compared to non-native second clutches ($p = 0.005$). Accordingly, mean egg mass was heavier in non-native first compared to non-native and native second clutches ($p = 0.01$ and 0.003, respectively).

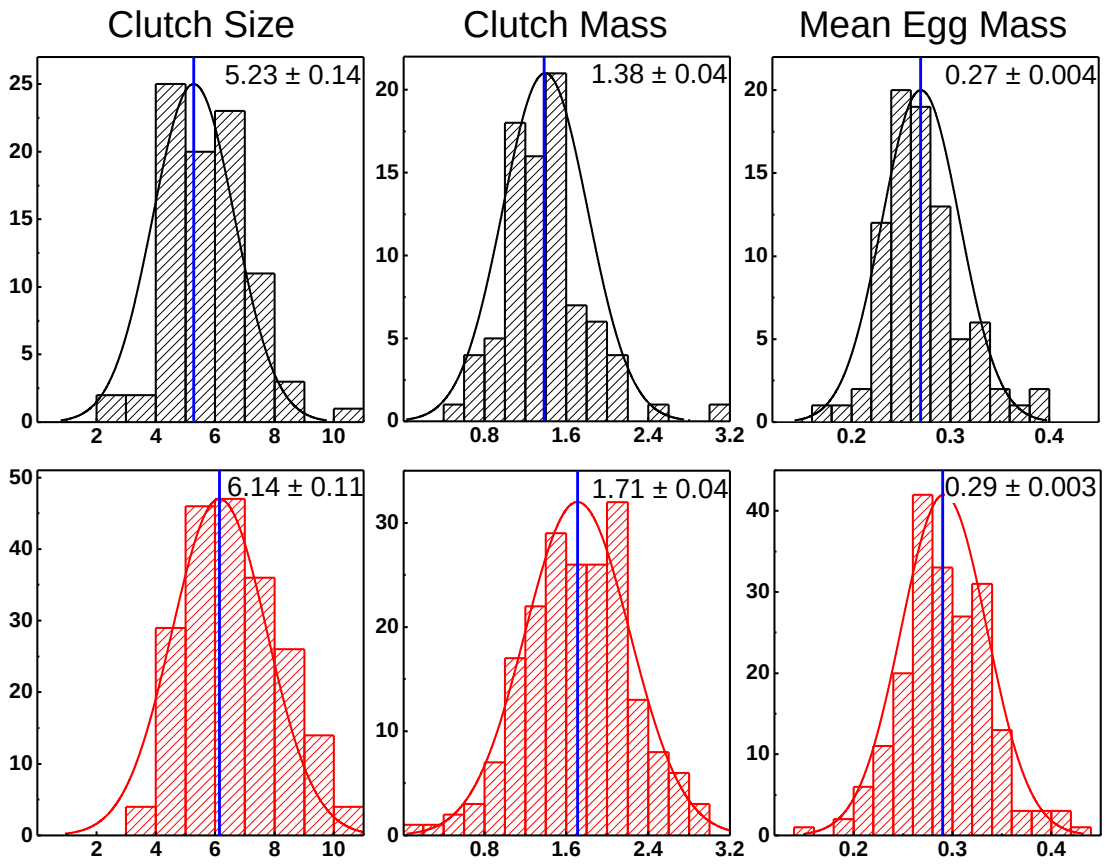

Figure 5.2: The distributions of clutch size, clutch mass (g), and mean egg mass (g) for the first seasonal clutches of native (above, black) and non-native (below, red) females collected between 2010 and 2015. The Y-axis of each plot depicts the counts of individuals and the solid blue line represents the mean value, which is reported (± 1 standard error) in the top right corner.

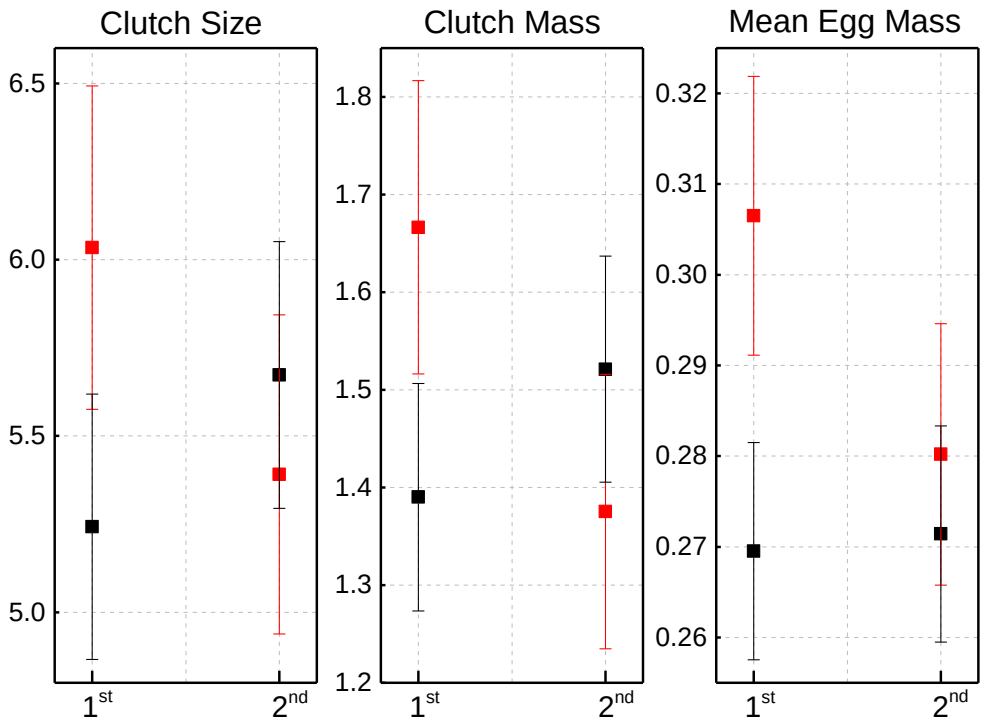

Figure 5.3: Interaction plots to show the effects of female origin (native - black or non-native - red) and seasonal clutch (1st or 2nd) on three measures of female reproductive investment (clutch size, clutch mass (g), mean egg mass (g)). Data are from females collected in 2014 and housed in experimental enclosures. Effect sizes are calculated from linear mixed models including female post-parturition body mass as a main effect and enclosure as a random effect. Error bars depict 95% confidence intervals.

Table 5.2: Summary statistics from tests for Origin differences in within-season female reproductive investment. Data are from females collected in 2014 and housed within experimental enclosures during their second seasonal receptive phase. Female ID nested within Population and Origin was included as a random effect in all models. Significant results are highlighted bold.

	Response	Origin	Clutch	Origin × Clutch	Post-parturition Body Mass
Female Reproductive Investment	Clutch Size	$F_{1,66} = 1.35$, $p = 0.24$	$F_{1,64} = 0.26$, $p = 0.61$	**$F_{1,66} = 6.56$, $p = 0.013$**	**$F_{1,94} = 40.94$, $p < 0.001$**
	Clutch Mass	$F_{1,69} = 0.69$, $p = 0.41$	$F_{1,60} = 2.21$, $p = 0.14$	**$F_{1,61} = 15.05$, $p < 0.001$**	**$F_{1,103} = 35.70$, $p < 0.001$**
	Mean Egg Mass	**$F_{1,69} = 7.73$, $p = 0.007$**	**$F_{1,60} = 5.33$, $p = 0.02$**	**$F_{1,60} = 6.95$, $p = 0.011$**	$F_{1,105} = 1.65$, $p = 0.20$

Male reproductive investment and sexual selection

Sexual Behaviour

Larger males engaged in more territorial interactions but there were no differences between native and non-native males in the numbers of competitive interactions observed (native: 14.49 ± 1.20, non-native: 13.93 ± 1.30; Origin: $\chi^2 = 0.06$, $p = 0.80$, Body Size: $\chi^2 = 45.55$, $p < 0.001$, Origin × Body Size: $\chi^2 = 1.30$, $p = 0.25$) or in the steepness of dominance hierarchies formed within each enclosure (Dominance Rank: $F_{1,56} = 321.44$, $p < 0.001$, Origin: $F_{1,9} = 0.71$, $p = 0.42$, Dominance Rank × Origin: $F_{1,56} = 1.33$, $p = 0.25$). Male body size predicted courtships, females courted and mating success, and Dominance predicted courtships and females courted; however, there were no significant differences in these relationships between native and non-native males (Tables 5.3 & 5.4). Overall, male-female courtship networks were significantly correlated with paternity networks within both native (Fishers Combined Test: $\chi^2 = 28.61$, $p < 0.001$, $df = 8$) and non-native (Fishers Combined Test: $\chi^2 = 29.9$, $p < 0.001$, $df = 10$) enclosures, and the range of effect sizes were similar for both origins (see Table S5.8 for matrix correlations by enclosure).

Table 5.3: Summary statistics from tests for Origin differences in the effects of male Body Size (standardized: mean = 0, SD =1) on three measures of male reproductive investment during the second seasonal reproductive episode. Enclosure was included as a random effect in all models. Results for main effects are reported from models excluding non-significant interaction terms. Significant effects are highlighted bold.

Response	Body Size	Origin	Origin × Body Size
Relative Number of Courtships	**$F_{1,56}$ = 9.27, p = 0.004**	$F_{1,7}$ = 0.07, p = 0.80	$F_{1,56}$ = 0.00, p = 0.99
Relative Number of Females Courted	**$F_{1,58}$ = 13.05, p < 0.001**	$F_{1,7}$ = 0.09, p = 0.77	$F_{1,56}$ = 0.52, p = 0.47
Relative Mating Success	**$F_{1,58}$ = 15.36, p < 0.001**	$F_{1,7}$ = 2.17, p = 0.19	$F_{1,56}$ = 0.41, p = 0.53

Table 5.4: Summary statistics from tests for Origin differences in the effects of male dominance (standardized: mean = 0, SD =1) on three measures of male reproductive investment during the second seasonal reproductive episode. Enclosure was included as a random effect in all models. Results for main effects are reported from models excluding non-significant interaction terms. Significant effects are highlighted bold.

Response	Dominance	Origin	Origin × Dominance	SVL
Relative Number of Courtships	**$F_{1,56} = 5.51$, $p = 0.022$**	$F_{1,7} = 0.06$, $p = 0.81$	$F_{1,59} < 0.01$, $p > 0.99$	**$F_{1,58} = 13.05$, $p < 0.001$**
Relative Number of Females Courted	**$F_{1,56} = 21.56$, $p < 0.001$**	$F_{1,7} = 0.11$, $p = 0.75$	$F_{1,59} = 0.10$, $p = 0.76$	$F_{1,61} = 2.30$, $p = 0.75$
Relative Mating Success	$F_{1,56} = 2.72$, $p = 0.10$	$F_{1,7} = 2.18$, $p = 0.19$	$F_{1,59} = 0.66$, $p = 0.42$	**$F_{1,61} = 17.81$, $p < 0.001$**

Opportunity and strength of sexual selection on males

There were no significant differences between native and non-native males in the estimated opportunity for sexual selection (Levene's Test on mating success: $F_{1,64} = 0.47$, $p = 0.49$, Table 5.5) or the opportunity for overall selection (Modified Levene's Test on fertilization success: $F_{1,64} = 0.36$, $p = 0.55$, Table 5.5). Similarly, there was no difference in the Bateman Gradient between native and non-native males (Table 5.5, Relative Mating Success: $F_{1,61} = 90.19$, $p < 0.001$, Origin: $F_{1,7} = 1.20$, $p = 0.31$, Origin × Relative Mating Success: $F_{1,61} = 0.11$, $p = 0.74$). Consequently, the estimated maximum intensity of selection (Jones Index) was similar for males of both origins (Table 5.5). Furthermore, there was little evidence for a relaxation of the relationships between male sexual traits and fertilization success in non-native males (Table S5.9). Levels of multiple paternity were similar within native and non-native enclosures (detected in 82% of native female clutches (Average Fathers: 2.39 ± 0.15) and 70% of non-native female clutches (Average Fathers: 2.44 ± 0.22): Origin: $\chi^2 = 0.00$, $p > 0.99$, Clutch Size: $\chi^2 = 0.85$, $p = 0.36$). Eleven percent of native males (4/38) and 10% of non-native males (3/29) sired no offspring during the experiment.

Table 5.5: Estimates of the opportunity for sexual selection (I_s), the opportunity for selection (I), the Bateman gradient (β_{ss}) and the maximum intensity of sexual selection (S'max) for native and non-native males housed within experimental enclosures during the second seasonal reproductive episode in 2014.

		Mating Success				Fertilization Success				Bateman Gradient		Max Intensity
	n	Mean (± 1 SE)	Var	I_s	$CI_{95\%}$	Mean (± 1 SE)	Var	I	$CI_{95\%}$	β_{ss}	$CI_{95\%}$	S'_{max}
Native	37	2.46 ± 0.24	2.20	0.36	0.22 0.65	5.11 ± 0.62	14.04	0.54	0.33 0.91	1.02	0.71 1.33	0.61
Non-Native	29	2.28 ± 0.27	2.06	0.40	0.24 0.74	5.00 ± 0.91	24.14	0.97	0.58 1.77	0.95	0.68 1.21	0.75

5.5 Discussion

Direct comparisons between ancestral and descendent populations living in different climates can help to reveal both the evolutionary potential of organisms and their limits to adaptation (Kawecki 2008). Consistent with adaptive responses to the low embryo survival prospects during the later stages of the breeding season (While *et al.* 2015), female wall lizards from non-native populations in England appear to shift their annual reproductive investment towards the first clutch of the season. Despite the low reproductive value of second clutches, both in terms of offspring number and low offspring survival, males from non-native populations invested in territoriality and mate acquisition during their second reproductive episode to the same extent as males from the native range. Taken together, our findings suggest greater constraints on adaptive shifts in male reproductive investment compared to females in response to cooler climate.

Non-native females produce more and larger eggs from their first compared to their second seasonal reproductive episode, and when compared to the first clutch of native females. This is consistent with latitudinal patterns of investment in lizards more generally, including European lacertids (Uller & While 2015). Furthermore, wall lizards from the northern range margin in Western Europe are less likely to lay second and third clutches compared to populations of the same lineage in southern France, and females from the lineage studied here (Aubret, Uller, & While, personal observations). This observation is indicative of female responses to seasonal time constraints on offspring survival. The greater maternal investment in first clutches observed in non-native wall lizards is plausibly an evolutionary response to strong selection for early season reproductive effort driven by direct climatic constraints on embryo development (While *et al.* 2015), and the survival advantage of larger offspring (Sinervo 1990). Heritability in both clutch size and egg size has indeed been demonstrated in natural lizard populations indicating the potential for rapid evolutionary responses in reproductive investment from

standing genetic variation (e.g. Sinervo & Doughty 1996; Sinervo & McAdam 2008). However, confirming the extent to which such variation in reproductive output represents genetic divergence between native and non-native populations requires the removal of environmental and maternal effects through long-term reciprocal transplant or common garden studies (e.g. Kaweck & Ebert 2004), which are logistically challenging to carry out in vertebrates.

Alternatively, but not necessarily exclusively, latitudinal shifts in reproductive investment could arise through phenotypic plasticity, initiated by environmental factors and their proximate effects on reproduction rather than adaptive genetic divergence. Climate is known to effect lizard growth and size at maturity, with cooler environments sometimes triggering larger adult body sizes at sexual maturity and, consequently, greater reproductive output (e.g. Wapstra & Swain 2001). This has been suggested to explain latitudinal variation in reproductive effort in common lizards, *Zootoca viviapara* (e.g. Roitberg *et al.* 2013). Nutritional state can be important for reproductive output (Olsson & Shine 1997b; Madsen & Shine 1999), thus our results may be a consequence of differences in nutritional availability between the native and non-native range and between the field verses laboratory populations. However, we found that the differences between native and non-native females in their within-season investment were independent of female post-parturition body mass. Furthermore, food availability in our enclosures during the time of the experiments was very high (males gained weight during the experiment, Table S5.10), suggesting that reduced investment in second clutches, as observed in non-native females, is unlikely to be a passive response to resource availability. Nevertheless, raising native and non-native individuals under different climatic conditions would be necessary to rule out that ontogenetic experiences drive the population differences we observed. Even if the divergence we observe is largely due to plasticity rather than a genetic response to selection, this shift in reproductive investment may provide an important source of adaptive variation following introduction, facilitating the future genetic adaptation of females to the new reproductive environment (West-Eberhard 2003; Uller & While 2015).

In our experimental populations, non-native females investing in larger and heavier first clutches also reduced their investment in second clutches, whereas native females exposed to the same conditions during their second reproductive episode maintained, or in some cases, increased their investment. A single episode of reproduction is likely to be the optimal investment strategy for non-native females. Indeed, we found a significantly lower incidence of second clutch production in non-native females (all native females produced a second clutch). Nevertheless, still relatively few non-native females (13%) refrained from producing a second clutch. This could be explained by a rarity of genetic variation for the physiological regulation of clutch production in the native range (typically three clutches per season in Italy, the source region of the non-native animals), which, in combination with the low founder numbers for the English populations, would constrain the evolutionary potential of clutch number in non-native populations.

Territoriality, mate searching, courtship and copulation are time-consuming and energetically costly for males (e.g. Merker & Nagy 1984; Shine & Mason 2005), and carry an increased risk of predation (Cooper Jr 1999). Therefore, there should be selection against male sexual behaviour when fitness returns are negligible (i.e. as for second clutches in England; While et al., 2015). Despite this, we found no experimental evidence that non-native males relaxed their behavioural investment in reproduction compared to males from the native range. There are several potential explanations. Firstly, selection may in fact maintain territoriality if quality resources are an important component of male survival at all times of the year and for the re-establishment of territory occupancy between seasons. However, this does not account for our observation that non-native male dominance scores remained strongly associated with courtship behaviour during the enclosure experiment, despite a predicted relaxation in this relationship. More likely, the maintenance of sexual behaviour is triggered by cue-response systems that have been reliable in past environments (e.g. Kriska *et al.* 2008; Sih 2013). Since

most species of lizard exhibit associated reproductive cycles whereby spermatogenesis, ovulation and mating occur synchronously (Méndez de la Cruz *et al.* 2015), the reproductive characters of the two sexes can be intimately linked. Consequently, courtship and mating often act as triggers for reproduction or receptivity in females (e.g. Crews *et al.* 1986; Manes *et al.* 2007), and male sexual activity can be primed by the presence of fertile females (e.g. Cooper & Perez-Mellado 2002; Head *et al.* 2005). If receptive females are the cues to which males respond there may be additional constraints on seasonal shifts in male behaviour if, as in wall lizards, females in non-native populations still reproduce second clutches (Crews & Moore 1986; also see Carretero 2006). Previous work has shown that second clutches are commonly fertile in non-native populations (Heathcote *et al.* 2016); hence, we did not expect to find infertility levels high enough for statistical testing. However, it is an interesting observation that, although rare, infertile eggs were more common in non-native females (25 % non-native vs 2% native of clutches had at least one infertile egg), which may indicate a lower mating rate or reduced sperm production by males (e.g. Olsson & Shine 1997a, Uller & Olsson 2005). Future work on the triggers of reproductive behaviour and its neuroendocrine basis would help to understand how the reproductive characters of the two sexes will coevolve across climatic regimes.

While environmentally driven shifts in female reproduction may relax selection on male sexual characters (e.g. Ibargüengoytía & Cussac 1999), we found no evidence that male sexual characters predict reproductive success to a greater or lesser extent in non-native populations, which corroborates our behavioural data. Furthermore, we found limited evidence for divergence in male phenotypes between native and non-native populations. The wall lizards used in this study belong to a geographically restricted lineage that show dramatic exaggeration of secondary sexual characters compared to lizards in other parts of the native range. This makes it difficult to make a meaningful comparison between males in non-native populations and populations at higher latitudes in the native range. Overall, our data may suggest that the overall intensity of selection on male traits is similar in the native and non-native populations,

despite differences in the seasonality of reproductive success. Nonetheless, sexual dimorphism is generally greater in non-native populations because of reduced trait expression in non-native females. Changes in the degree of sexual dimorphism are often attributed to sexually selected exaggeration in males (Andersson 1994), but increased dichromatism due to loss of female ornamentation is supported by phylogenetic studies on birds (e.g. Burns 1998; Wiens 2001; Hofmann *et al.* 2008) and in dragon lizards (Ord & Stuart-Fox 2006). The causes of reduced expression in female colouration, head size and bite force in non-native populations could involve relaxed female-female competition or, at least for colouration, increased importance of crypsis. The relative contribution of these factors cannot be untangled by the present study but this would represent an interesting avenue for future research.

In summary, we demonstrate adaptive within-season shifts in female reproductive investment in wall lizards following their recent introduction to cooler climates. Both plasticity and genetic divergence may account for these patterns. In contrast, we found no experimental evidence to suggest a corresponding loss of male investment in reproduction later in the season in non-native populations. The ability of female and male reproductive investment strategies to respond adaptively over short or long time scales will depend upon how responses in one sex affect the reliability of cues in the other. We suggest that associated reproductive cycles between males and females play a role as constraints on adaptive shifts in male behaviour.

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5.8 Supplementary Figures

Figure S5.1: Photograph of outdoor enclosures used to house lizards during the 2014 experiment.

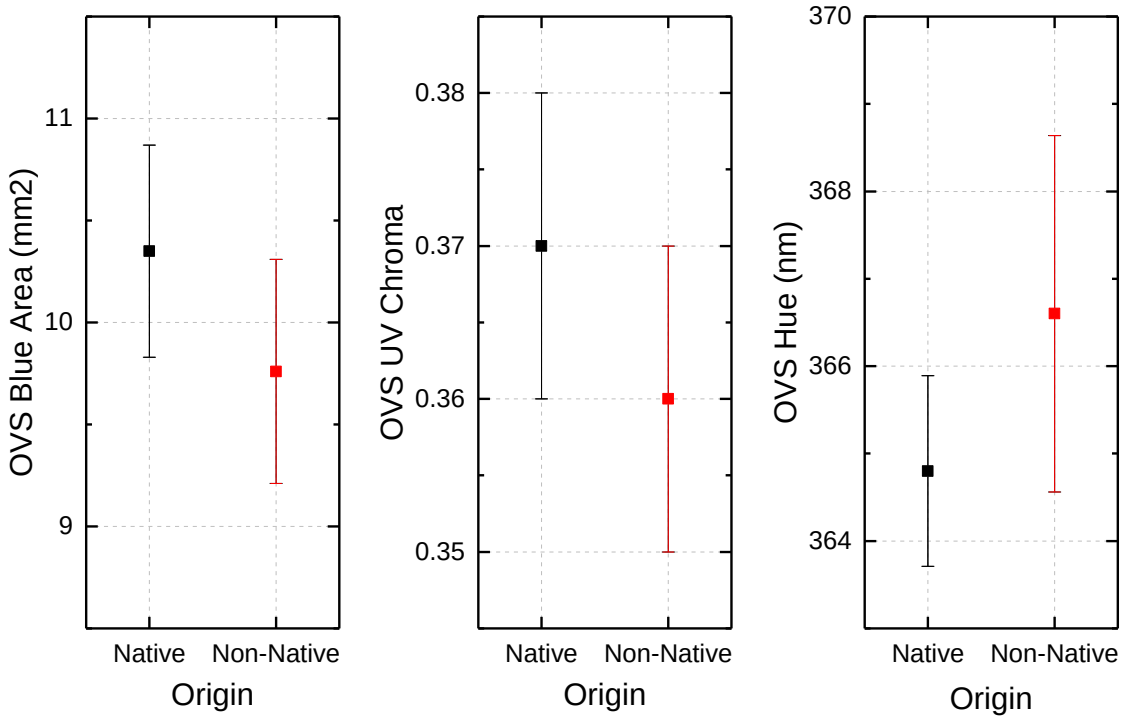

Figure S5.2: Outer ventral scale traits (area of blue colouration (OVS blue area) and spectral reflectance (OVS UV Chroma and OVS Hue)) expected to be under intra-sexual selection in Podarcis lizards. Values depict the mean (± 1SE) for native and non-native males, respectively. See Table S1 for sample sizes.

5.9 Supplementary Tables

Table S5.1: Details of the native and non-native populations included in this study. Year of capture and the sample sizes of males and females are reported for each population. Haplotype abbreviations correspond to Tuscan (TUS) and Venetian (VEN). Populations sampled for the 2014 enclosures experiment are highlighted in grey.

Population	Country	Origin	First Record	Abbreviation	Latitude	Longitude	Altitude	Haplotype*	Year(s) of Capture	No. Females	No. Males
Bacchereto	Italy	Native	N/A	BC	43.81	10.99	217	TUS	2015	4	12
Castelarrano	Italy	Native	N/A	CT	44.51	10.73	157	VEN	2013	7	20
Castelfiorentino	Italy	Native	N/A	CL	43.61	10.97	64	TUS	2014	5	7
Castellacio	Italy	Native	N/A	CS	43.49	10.36	281	TUS	2012	1	3
Castelnuovo Berardenga	Italy	Native	N/A	CB	43.34	11.50	337	TUS	2013	4	5
Certaldo	Italy	Native	N/A	CD	43.55	11.04	76	TUS	2014-2015	6	12
Chianni	Italy	Native	N/A	CN	43.48	10.64	297	TUS	2013, 2015	12	36
Colle di Val' Elsa	Italy	Native	N/A	VE	43.42	11.11	229	TUS	2013-2015	20	26
Crespina	Italy	Native	N/A	CR	43.57	10.56	80	TUS	2012	7	15
Greve in Chianti	Italy	Native	N/A	GC	43.59	11.31	227	TUS	2013 - 2015	41	27
Montemassi	Italy	Native	N/A	MM	42.99	11.06	240	TUS	2013	14	8
Montecatini Alto (Terme)	Italy	Native	N/A	MT	43.89	10.79	282	TUS	2015	5	16
Nonantola	Italy	Native	N/A	NO	44.68	11.04	26	VEN	2013	12	16
Peccioli	Italy	Native	N/A	PE	43.54	10.72	127	TUS	2014-2015	11	32
Pian Di Venola	Italy	Native	N/A	PV	44.33	11.19	145	VEN	2012	10	12
Prato	Italy	Native	N/A	PR	43.9	11.11	86	TUS	2013	16	14
Travale	Italy	Native	N/A	TR	43.17	11.01	509	TUS	2013	9	13
Vignola	Italy	Native	N/A	VG	44.47	11.01	121	TUS, VEN	2013	12	8
Dancing Ledge	UK	Non-Native	1990	DL	50.59	-2.01	19	VEN	2009-2011	38	27
Folkestone	UK	Non-Native	1992	FS	51.09	-1.20	96	VEN	2009-2011	12	7
Newton Ferrers	UK	Non-Native	1978	NF	50.32	-4.04	49	VEN	2009-2011	14	11
Seacombe	UK	Non-Native	1986	SC	50.59	-2.02	19	VEN	2009-2011	8	6
Shoreham	UK	Non-Native	1975	SH	50.83	-0.26	5	VEN	2009-2011	55	36
Shorwell	UK	Non-Native	1985	SW	50.65	-1.36	70	TUS, VEN	2009-2011	35	18
Ventnor Botanical Garden	UK	Non-Native	2000	VB	50.59	-1.23	24	TUS, VEN	2009-2014	45	37
Ventnor Town	UK	Non-Native	1930	VT	50.59	-1.21	28	TUS	2009-2014	112	82
West Worthing	UK	Non-Native	2004	WW	50.82	-0.39	6	VEN	2009-2011	24	15
Winspit	UK	Non-Native	1986	WS	50.58	-2.03	11	VEN	2009-2014	29	44

*Mitochondrial sequences analysed by Michaelides et al. 2015. Molecular Ecology.

Table S5.2: Mean trait values (± 1SE) and sample sizes for native and non-native lizards.

	Females				Males			
	Native		Non-native		Native		Non-native	
	n	mean (± 1 SE)	n	mean (± 1 SE)	n	mean (± 1 SE)	n	mean (± 1 SE)
SVL (mm)	196	58 ± 0.33	372	61 ± 0.26	282	61 ± 0.35	283	62 ± 0.25
Head Length (mm)	196	12.9 ± 0.08	372	12.9 ± 0.04	282	15.5 ± 0.11	283	15.6 ± 0.07
Blackness (%)	126	27 ± 1.24	179	28 ± 1.07	127	46 ± 1.21	143	50 ± 1.22
Greenness	195	5 ± 0.20	365	5 ± 0.13	281	7 ± 0.15	268	7 ± 0.13
Body Mass (g)	186	4.56 ± 0.08	225	5.35 ± 0.08	277	5.94 ± 0.10	142	5.96 ± 0.10
Bite Force (N)	58	2.72 ± 0.10	31	2.60 ± 0.14	90	7.37 ± 0.34	32	7.86 ± 0.40
Blue Spot Area (mm^2)	124	3.56 ± 0.34	134	1.70 ± 0.13	124	10.34 ± 0.52	113	9.76 ± 0.55
OVS UV Chroma					66	0.37 ± 0.01	28	0.36 ± 0.01
OVS Hue (nm)					66	364.8 ± 1.09	28	366.6 ± 2.04

Table S5.3: Factor loadings for male body size, the first principal component based on the male traits SVL, head length, head width and mass.

	PC1 loadings			
Proportion variance	SVL	Head Length	Head Width	Mass
0.96	0.93	0.23	0.13	0.26

Table S5.4: Details on the 16 microsatellites used for the analysis of offspring paternity. Primers were combined within five multiplexes.

Multiplex*	Locus		Primer sequence (5'-3')	Product size (bp)	Repeat motif	Range (bp)
1	PmurC150	F	[6-FAM]GTCAGCTTTGCAGCACCTTAG	193	CA	171-217
		R	GCGATTAGAGAAGGCGTTTG			
	PmurC168	F	[HEX]GGTCCGGCTTCAAAGAATAAG	244	TTTC	210-306
		R	CAGAGGACTCGCTCAAGGAC			
	PmurC275_278	F	[6-FAM]GCTTAAAATTAATGCTGCTATTGTATC	245	TATC	219-610
		R	ATAGGTAGAAAATTTATAAACCCTTGG			
2	PmurC164	F	[6-FAM]ATCGATGAATGAAGGGCAGT	216	GATA	170-246
		R	CCAGGCATTGTCAAACTATCTG			
	PmurC038	F	[HEX]CAATGTGCAGTGTTGGGTTG	210	TATC	193-425
		R	ATGTGAGCGACTCCTGGATG			
	PmurC028	F	[6-FAM]TTGCTTCTGAATACGCCTAGC	287	TATC	253-543
		R	AGTGTATTGCGACTGTCAATGG			
3	PmurC356	F	[6-FAM]GATCTTCAGATGAAGGGTAGTTAGAT	159	GTTA	138-178
		R	ATGAAGACAAACAGGCTTGG			
	PmurC109	F	[HEX]AGGAGCCCAGCAGCTGAA	309	GTA	295-355
		R	TTTACATAGACCTGCGGGTATGG			
	PmurC103	F	[6-FAM]CCAGGTCTTGTGATCGAGTG	350	GATA	316-480
4	Pm01	F	[6-FAM] CCACAGGCATCTGGTTAG	128	$(ATT)_{16}$	119-137
		R	TCCATAAGACTGTAAGACAAGCC			
	Pm05	F	[HEX] CAAGAGGGCAGCCTAGTAATG	160	$(AGAT)_{10}$	135-185
		R	AGATGGGCTCATTTCAACTCC			
	Pm09	F	[NED] ACGTGTTTCTGTGCTTTGC	189	$(ATT)_{17}$	176-203
		R	AGTCAGACGAGAGGTTGCC			
	Pm16	F	[6-FAM] GGGATGGAGAAAGATGGCG	192	$(TCTT)_{16}$	179-211
		R	GCACTTGCCTACTGGTCATAC			
5	Pm02	F	[HEX] TTGGGAAGAAGGGGAAGGG		$(AACC)_7$	164-216
		R	ATGGCCGCTAGGTCAAGTG			
	Pm19	F	[6-FAM] CAGCCACAAGGTGAACCAG		$(AGGC)_{11}$	164-204
		R	TGTGAGGTCAGAGGCATGG			
	Pm14	F	[NED] GCAGGATCAGAGCGCAATC		$(GCAG)_7$	151-187
		R	TGTGGCATGTTGAGACACC			

*Mulitplexes 1, 2 and 3 were developed by Heathcote et al. 2015. Conservation Genetic Resources. Multiplex 4 and 5 were developed by Richards et al. 2012. Molecular Ecology Resources.

Table S5.5*:* Pearson's correlation coefficients between phenotypic traits reported separately by origin (native (bottom triangle) and non-native (top triangle)) and sex (males (left) and female (right)). Data are from lizards used in the 2014 enclosure experiment.

Males	SVL (mm)	Body Mass (g)	Head Length (mm)	Greenness	Blackness (%)	Blue Spot Area (mm^2)	Bite Force (N)	OVS Hue (nm)	OVS UV Chroma	Females	SVL (mm)	Body Mass (g)	Head Length (mm)	Greenness	Blackness (%)	Blue Spot Area (mm^2)	Bite Force (N)
SVL (mm)		0.83	0.82	0.22	0.22	0.25	0.19	0.29	-0.14	SVL (mm)		0.76	0.69	0.26	0.33	-0.05	0.77
Body Mass (g)	0.89		0.82	0.41	0.16	0.25	0.32	0.36	-0.09	Body Mass (g)	0.91		0.67	0.19	0.38	-0.16	0.54
Head Length (mm)	0.91	0.93		0.35	0.22	0.25	0.37	0.44	-0.20	Head Length (mm)	0.86	0.82		0.30	0.34	0.08	0.62
Greenness	0.64	0.68	0.70		0.05	0.20	0.37	0.37	-0.20	Greenness	0.68	0.61	0.67		0.60	0.17	0.22
Blackness (%)	0.37	0.26	0.38	-0.01		-0.28	-0.21	0.36	-0.30	Blackness (%)	0.44	0.31	0.33	0.34		0.14	0.32
Blue Spot Area (mm^2)	0.37	0.37	0.49	0.37	0.05		0.08	-0.06	0.30	Blue Spot Area (mm^2)	-0.04	-0.06	-0.14	-0.12	-0.03		0.05
Bite Force (N)	0.83	0.83	0.87	0.55	0.31	0.34		-0.10	0.28	Bite Force (N)	0.68	0.62	0.61	0.34	0.35	-0.20	
OVS Hue (nm)	0.31	0.40	0.20	0.26	0.01	-0.05	0.27		-0.62								
OVS UV Chroma	0.29	0.17	0.37	0.19	0.34	0.46	0.26	-0.36									

Table S5.6: Results from tests for divergence in first clutch reproductive investment (clutch size, clutch mass and mean egg mass) for native and non-native females sampled between 2010 and 2015. Models were run controlling for post-parturition body mass (above) and excluding post-parturition body mass (below). Source population nested within origin was included as a random effect in all models. Significant effects are highlighted bold.

	Response	n	Origin	Post-parturition body mass	Year	SVL
	Post-parturition Body Mass	296	**$F_{1,19}$ = 5.59, p = 0.03**		**$F_{5,268}$ = 273.46, p < 0.001**	**$F_{1,287}$ = 4.78, p < 0.001**
Models controlling for post-partum mass	Clutch Size	296	**χ^2_1 = 5.39, p = 0.02**	**χ^2_1 = 26.17, p = 0.02**	χ^2_5 = 5.82, p = 0.32	
	Clutch Mass	283	**$F_{1,13}$ = 15.53, p = 0.002**	**$F_{1,195}$ = 111.89, p < 0.001**	**$F_{5,138}$ = 5 .95, p = 0.003**	
	Mean Egg Mass	283	$F_{1,13}$ = 1.93, p = 0.19	**$F_{1,195}$ = 9.25, p = 0.003**	**$F_{5,138}$ = 3.37 , p = 0.007**	
Models excluding post-partum mass	Clutch Size	296	χ^2_1 = 5.57, p = 0.06		χ^2_1 = 5.28, p = 0.38	
	Clutch Mass	283	**$F_{1,17}$ = 5.59, p = 0.03**		**$F_{5,219}$ = 3 .11, p = 0.01**	
	Mean Egg Mass	283	$F_{1,12}$ = 1.58, p = 0.23		**$F_{5,154}$ = 3.49 , p = 0.005**	

Table S5.7*:* Results from tests for differences between native and non-native experimental females in their within-season patterns of reproductive investment. Models were run excluding post-parturition body mass (above) and replacing post-parturition body mass with SVL (below) to demonstrate the consistency of effects reported in Table 5.2 of the main manuscript. Female ID nested within Population and Origin was included as a random effect in all models. Significant results are highlighted bold.

	Response	Origin	Clutch	Origin × Clutch	SVL
Models excluding post-partum mass	Clutch Size	$F_{1,67} = 5.47$, p = 0.02	$F_{1,64} = 0.02$, p = 0.89	$F_{1,63} = 2.98$, p = 0.09	
	Clutch Mass	$F_{1,68} = 2.75$, p = 0.10	$F_{1,57}$=2.98, p = 0.17	**$F_{1,59} = 14.89$, p < 0.001**	
	Mean Egg Mass	**$F_{1,68} = 13.08$, p < 0.001**	**$F_{1,61} = 6.98$, p = 0.01**	**$F_{1,61} = 8.01$, p < 0.001**	
Models controlling for SVL	Clutch Size	$F_{1,64} = 0.00$, p = 0.95	$F_{1,65} = 0.01$, p = 0.94	**$F_{1,65} = 5.39$, p = 0.02**	**$F_{1,81} = 74.69$, p < 0.001**
	Clutch Mass	$F_{1,66} = 0.34$, p = 0.56	$F_{1,60} = 2.74$, p = 0.14	**$F_{1,59} = 18.00$, p < 0.001**	**$F_{1,75} = 66.83$, p < 0.001**
	Mean Egg Mass	**$F_{1,66} = 8.29$, p = 0.005**	**$F_{1,61} = 7.29$, p = 0.009**	**$F_{1,61} = 8.89$, p = 0.004**	$F_{1,76} = 2.43$, p = 0.12

Table S5.8: Correlations between male-female courtship networks and paternity networks from the nine experimental enclosure populations. Combined test statistics are reported for native and non-native enclosures, respectively.

	Matrix Correlations By Enclosure			Fisher's Combined Test				
	Enclosure	Matrix Correlation (R_M)	p	Logged	df	Sum	X^2	p
Native	1	0.193	0.144	-1.94				
	3	0.268	0.049	-3.02				
	7	0.627	0.002	-6.32				
	8	0.141	0.151	-1.89				
	9	0.157	0.16	-1.78	10	-14.95	29.91	0.0009
Non-Native	2	0.2	0.096	-2.34				
	4	-0.003	0.406	-0.9				
	5	0.262	0.052	-2.95				
	6	0.623	0	-8.11	8	-14.3	28.61	0.0004

Table S5.9: Results from tests for differences between native and non-native males in the slope of the relationship between seven known or putative sexually selected traits and relative fertilization success during the enclosures experiment. Results for main effects are reported from models excluding non-significant interaction terms. Traits were standardized (mean = 0, SD = 1) prior to analysis and SVL was included as a covariate. Regression estimates (± 1 SE) for traits from analyses performed separately by origin are also reported.

Trait	Native	Non-native	Origin	Trait	Origin × Trait	SVL
PC1_BodySize	0.47 ± 0.10	0.42 ± 0.15	$F_{1,7} = 0.04$, p = 0.85	**$F_{1,58} = 25.57$, p < 0.001**	$F_{1,57} = 0.08$, p = 0.78	
Greenness	-0.07 ± 0.14	0.21 ± 0.17	$F_{1,6} < 0.01$, p = 0.95	$F_{1,56} = 0.55$, p = 0.46	$F_{1,55} = 1.01$, p = 0.32	**$F_{1,54} = 13.54$, p < 0.001**
Blackness	0.13 ± 0.11	0.12 ± 0.16	$F_{1,6} = 0.04$, p = 0.85	$F_{1,59} = 2.85$, p = 0.10	$F_{1,56} = 0.02$, p = 0.88	**$F_{1,55} = 16.87$, p < 0.001**
Blue Spot Area	0.01 ± 0.13	-0.25 ± 0.16	$F_{1,6} < 0.01$, p = 0.94	$F_{1,56} = 1.16$, p = 0.29	$F_{1,55} = 1.87$, p = 0.18	**$F_{1,56} = 21.31$, p < 0.001**
Bite Force	0.05 ± 0.18	-0.16 ± 0.16	$F_{1,7} = 0.06$, p = 0.82	$F_{1,62} = 0.80$, p = 0.37	$F_{1,60} = 0.69$, p = 0.41	**$F_{1,60} = 17.97$, p < 0.001**
OVS UV Chroma	0.07 ± 0.12	-0.07 ± 0.16	$F_{1,6} < 0.01$, p = 0.94	$F_{1,57} = 0.02$, p = 0.88	$F_{1,56} = 0.54$, p = 0.47	**$F_{1,56} = 19.83$, p < 0.001**
OVS Hue	-0.02 ± 0.12	0.08 ± 0.17	$F_{1,6} < 0.01$, p = 0.94	$F_{1,54} = 0.08$, p = 0.77	$F_{1,52} = 0.14$, p = 0.71	**$F_{1,56} = 17.54$, p < 0.001**

Table S5.10: Mean (± 1 SE) initial capture mass, enclosures release mass, and enclosures recapture mass (grams) reported by Sex and Origin. Data exclude the lizards that were not recaptured from the enclosures.

Sex	Origin	Initial Capture Mass	Enclosures Release Mass	Enclosures Recapture Mass
Males	Native	6.15 ± 0.26	6.41 ± 0.26	6.61 ± 0.26
	Non-Native	6.02 ± 0.22	6.00 ± 0.20	6.13 ± 0.21
Females	Native	4.71 ± 0.18	4.65 ± 0.14	5.41 ± 0.17
	Non-Native	6.02 ± 0.21	5.03 ± 0.16	5.97 ± 0.25

5.10 Supplementary Information

Additional information on the quantification of morphological traits

i) **Greenness**: where greenness scores conflicted between the two scorers, we retained the mean value for analyses. Subjective Greenness scores are highly correlated with scores from digital photographs analysed in Photoshop CS4 and with values for green chroma extracted using spectrophotometry (While *et al.* 2015).

ii) **Blackness**: we estimated % ventral blackness from the chest area, by quantifying the proportion of black to non-black pixels in the computer program Image J (Abràmoff *et al.* 2004), using ventral surface photographs of each lizard taken upon capture. Since chest blackness is highly correlated within individuals with blackness on the throat and stomach (While *et al.* 2015), this score was therefore considered representative of blackness on an individual's overall ventral surface (see While et al. 2015 for full details of method).

iii) **OVS Blue Area**: for a sample of individuals we estimated the absolute area of blue colouration on their outer ventral scales of their left lateral side (i.e. blue spots) from photographs taken upon capture. In the program Image J, a scaled object in each photograph was used to set the scale for the image and the polygon tool was used to manually trace around the blue areas on the scales to estimate their overall area.

iv) **Bite Force**: we recorded bite force to the nearest 0.01 Newtons using a custom-made bite force meter. We conducted three successive trials and retained the largest maximum bite force recording as the representative measure for use in analyses (While *et al.* 2015). Each animal was tested in the middle of the lab light cycle, to maximise the likelihood that they had reached their optimal body temperature, however, to control for variation in body temperature at the

time of testing, we also recorded the skin surface temperature of each individual at the time of testing using an infrared duel laser digital thermometer.

v) **OVS UV Chroma and OVS Hue**: we obtained reflectance spectra from the outer ventral scales (OVS) of a sample of males (see Pérez i de Lanuza *et al.* 2014 for methods). Where possible we selected the second and third OVS from each side of the male. From the reflectance spectra we calculated OVS UV Chroma, the proportion of reflectance in the UV-blue spectrum to that of the visible spectrum ($R_{300\text{-}400}/R_{300\text{-}700}$) and OVS Hue, the wavelength at maximum reflectance. From each male, we retained the average UV Chroma and Hue scores for the analyses.

Additional information on the outdoor enclosure experiment

i) **Enclosures Set-up**: within each enclosure, we created a gradient in habitat complexity by constructing three types of sites that varied in both structural complexity and the opportunity for thermoregulation. Each site consisted of two stacked pallets (1.14 m^2) sandwiched with a sheet of felt underlay, but varied in the number and construction of concrete breezeblocks placed above the pallets, which acted as both shelter and as a thermal resource. We arranged high, medium and low quality pallets in a three by three organization from one side of the enclosure to the other (see Figure S5.1).

ii) **Assignment to enclosures:** to reduce population of origin effects on mating behaviour and reproductive investment, each enclosure housed lizards sourced from a minimum of three different populations within each sex. Within this constraint, we assigned males and females to the enclosures randomly within further constraints set by when females' laid their first clutches. There was no significant difference among enclosure populations in the SVL of males (Enclosure: $F_{8,63} = 0.08$, $p = 1.00$). However, consistent with divergence in female body size between native and non-native populations, there was a significant difference among enclosure populations in the SVL of females (Enclosure: $F_{8,63} = 2.60$, $p = 0.016$), with non-native females

larger on average (native enclosure females: 58.6 ± 0.81, non-native enclosure females: 62.6 ± 0.80).

iii) **Interaction Data Collection:** to quantify the number, direction and outcome of interactions between individuals, one observer (HEAM) carried out 45 minute periods of observations on each of the enclosures. Daily behavioural observation periods began when we observed the first lizards in the morning and ended at dusk. The sequence of enclosures during interaction data collection remained the same throughout the experiment; however, we rotated the first enclosure of the round between days to avoid a temporal effect on observations.

iv) **Classification of social interactions:** we recorded the identity of interacting lizards, the initial location of the receiver, and the nature of the social interaction according to an ethogram (Heathcote *et al.* 2016). We classified behavioural interactions into three categories: male-male agonistic, male-female courtship and other. Male-male agonistic interactions included behaviours such as chases, physical attacks and aggressive posturing between males. To distinguish these interactions from non-combative male-male behaviour, we only classified interactions as male-male competition when they included a submissive behaviour by one male in the presence of another (i.e. a retreat) and this determined which male was deemed the "winner" of the encounter. We used this outcome to generate normalised male dominance scores (David 1988), on the basis of dyadic dominance indices (Dij), in which the observed proportion of wins was corrected for chance occurrence (de Vries *et al.* 2006). Prior to testing for Origin differences in the steepness of male dominance hierarchies we confirmed that all enclosure hierarchies were significantly linear in form by calculating the slope from a linear regression between normalised dominance score (Dominance) and Dominance Rank (1-8, most dominant male to least within and enclosure), for every enclosure, which we simulated over 10,000 iterations (CRAN: Package Steepness, de Vries *et al.* 2006).

We classified male-female interactions as courtships when they included display behaviour from a male directed towards a female or a tail grab by the male. We deemed these behaviours indicative of male sexual interest in a female or intention to mate.

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Chapter 6

General Discussion

In this thesis I examined (i) the causes and consequences of sexual selection in secondary contact between lineages that differ in secondary sexual characters (**Chapters 2-4**), and (ii) responses in female reproductive investment and male sexual strategies following introduction to a cooler and more seasonal environment with strong constraints on the seasonal timing of reproductive success (**Chapter 5**). These investigations offered differing perspectives on the contribution of sexual selection and sexually selected characters to rapid and adaptive phenotypic change. In this chapter I summarise the main findings within the context of our current understanding of how sexual selection may shape the fate and characteristics of regions of secondary contact and how life-history and sexually selected behaviour may respond to environmental change. I also provide some reflections on the consequences of my results for broader patterns of introgression and speciation and suggest future research directions in relation to this work.

6.1 The consequences of sexual selection following secondary contact

Male-male competition can have a direct role in promoting rapid and adaptive introgression. Introgression is increasingly recognized as an important source of evolutionary change in animals (Hedrick 2013; Rius & Darling 2014; Tigano & Friesen 2016), and its consequences for adaptation through natural selection is well characterized in mice, *Heliconius* butterflies, humans, and mosquitos (e.g. Song et al. 2011; Pardo-Diaz et al. 2012; Huerta-Sanchez et al. 2014; Norris et al. 2015). However, traits that confer an advantage in acquiring and fertilizing mates should also play a fundamental role in this process. As a result, an increasing number of studies have sought to characterize sexual selection regimes in zones of secondary contact. Undoubtedly, the best evidence for adaptive introgression in the context of sexual selection has come from studies of female mate choice in response to divergent male sexual signals (Parsons *et al.* 1993; Stein & Uy 2006; Baldassarre & Webster 2013; Baldassarre *et al.* 2014). This focus on female choice can partly be attributed to the overwhelming focus on female choice in models of speciation (e.g. Lande 1981; Dieckmann & Doebeli 1999; Higashi *et al.* 1999; Kondrashov & Kondrashov 1999; Kawata & Yoshimura 2000), and the fact that female choice is often a prominent evolutionary force in the kinds of taxa that are typically studied in the context of hybridization and introgression (e.g. birds). However, this thesis suggests that male-male competition may play just as an important role. Indeed, **Chapters 2-4** present some of the best evidence yet that behaviours and traits associated with male-male competition can cause highly directional hybridization, drive patterns of gene transfer in regions of secondary contact, and contribute to the adaptive spread of sexually selected male phenotypes. This may be the case in lizards in particular because male contest competition is considered the primary cause of the evolution of male sexual characters (e.g. Ord *et al.* 2001; Cox *et al.* 2003; Pérez i de Lanuza *et al.* 2013) with a lesser role identified for female mate choice, at least in terms of pre-copulatory mate preferences (Olsson & Madsen 1995). Thus, it seems reasonable to predict that these effects should be common following secondary contact between lizard lineages,

particularly when differences between lineages in the sexual phenotypes of males are pronounced. In support of this, other studies of secondary contact between morphologically divergent lizard lineages have similarly reported patterns of asymmetric introgression (Olave *et al.* 2011; Jezkova *et al.* 2013; Robbins *et al.* 2013). However, in only one of these examples, hybridization between the Eastern Fence Lizard, *Sceloporus undulatus*, and the Florida Scrub Lizard, *S. woodi*, were asymmetries in competitive ability identified as driving patterns of gene flow. Sexual selection is expected to have greater importance when compared to other mechanisms (e.g. genetic incompatibilities, natural selection) for patterns of hybridization and introgression in more closely related lineages. Thus, consistent effects of male contest competition and asymmetries in male competitive ability in shaping the degree, direction, and targets of genetic and phenotypic introgression in lizards may be most likely to be found in the context of intraspecific hybridization.

Less dominant Italian males appear more prone to hybridize. **Chapter 3** revealed a layer of complexity to the proximate causes of hybridization between Italian males and Western European females. First, there was evidence for assortative courtship and mate preferences in males of the Italian lineage. Second, dominance strongly predicted male reproductive success within the Italian lineage (as would be expected under strong male-male competition). Third, putative selection gradients on the visual characters of Italian males via hybridization were weak (and for some traits reversed in direction) when compared to selection via within-lineage reproductive success. These results suggested that the subdominant Italian males may be more prone than the dominant Italian males to mate with Western European females in a mixed-lineage social context. The outcome is similar to a 'best-of-a-bad-job' strategy under circumstances where conspecific mates are rare because of biased sex ratios or low population densities (e.g. Hubbs 1955). Indeed, phenotype-dependent hybridization seems to be common, with matings often occurring between individuals with features that make them less competitive or attractive to conspecifics (e.g. Baker 1996; Nuechterlein & Buitron 1998; Veen *et*

al. 2001; Randler 2006). Nonetheless, the experimental results, combined with evidence of limited fitness costs to hybridization (in terms of hybrid viability and fertility), predicted that introgression would be asymmetric following secondary contact, occurring from the Italian into the Western European lineage. Indeed, both predictions were borne out in three independent zones of secondary contact. Although hybridization via subdominant Italian males will not affect the directionality of gene flow it could have consequences for the rates at which different phenotypic characters introgress. However, it is important to note that the patterns of hybridization and evidence for the strength and targets of sexual selection on Italian and Western European males reported in this thesis come from the examination of male reproductive success under experimental mixed-lineage conditions. Therefore, how well these data reflect the strength and direction of hybridization and sexual selection in real zones of secondary contact is unknown. Moreover, the experimental populations only simulated initial contact between the lineages. Because introgression occurs in the next or later generations it would be interesting to study the heritability of male sexual characters in first generation hybrid offspring and hybrid fitness in admixed populations. Detailed documentation of male sexual characters, behaviour and reproductive success in naturally hybridizing Italian and Western European lizards and their offspring could offer insights into the effects of the mating patterns of dominant and subdominant Italian males on rates of introgression. This could potentially also reveal the causes of differential rates of introgression in visual and chemical characters (**Chapter 4**). It would also be interesting to study the dispersal behaviour of Italian lizards at the leading edge of the hybrid zone. If dominant Italian males or juveniles that are more likely to become the most dominant males are more prone to disperse and colonize new environments (e.g. Duckworth 2006), this may exaggerate asymmetric introgression. Alternatively, if subdominant Italian males are more prone to disperse under natural conditions (e.g. because they are unable to secure a territory) then rates of phenotypic introgression may be slowed down, at least initially, following secondary contact.

The consequences for asymmetries in male-male competition following secondary contact may also have broader implications for patterns of diversification. Introgression and speciation are fields of research that largely have developed independently from one another despite being united by similar themes, including sexual selection, hybridization, and reproductive isolation. There is increasing evidence that the former may lead directly to the latter (e.g. Stemshorn *et al.* 2011) and that inter-specific hybridization and introgression can occur despite strong pre- and post-zygotic reproductive barriers (e.g. Sambatti *et al.* 2012). Thus, conceptually, it may be useful to consider hybridization and introgression as variable processes on a continuum towards reproductive isolation and speciation with the knowledge that gene flow can continue after species divergence (Mallet *et al.* 2007; Hochkirch 2013).

Until recently, the topic of speciation has motivated more research explicitly investigating the fitness outcomes of female sexual behaviour following secondary contact rather than male competition, probably since assortative female mate preferences for divergent male traits make a more transparent contribution to reproductive isolation (e.g. Hoskin *et al.* 2005; Mendelson & Shaw 2005). However, there have been recent calls for a shift in perspective towards integrating male competition into a more holistic understanding of speciation (Qvarnström *et al.* 2012; Lackey & Boughman 2013). Indeed, male-male competition has the potential to contribute significantly to patterns of hybridization, introgression, and new species formation in a broad range of taxonomic groups. For example, male-male competition may maintain colour polymorphisms in signalling traits with dual functions in mate choice via negative frequency-dependent selection, and thus set the stage for future reproductive isolation and speciation if females evolve alternative preferences for colour morphs (Seehausen & Schluter 2004; van Doorn *et al.* 2004). This could be a mechanism for reproductive isolation, and hence speciation, among taxa where males behave less aggressively towards competitors with a rare or dissimilar colour morph, including, for example, between haplochromine cichlids (Dijkstra *et al.* 2007), *Calopteryx* damselflies (Tynkkynen *et al.* 2005), and flycatchers (Vallin *et al.* 2012). However,

asymmetries within and between lineages in the outcomes of male contests, delivery of aggression by males or responses to aggression also have the potential to affect patterns of hybridization and introgression. For example, in *Calopteryx* damselflies, males of one species also display alternative mating tactics, and the highly ornamented and territorial males have a tendency to hybridize (Tynkkynen *et al.* 2009). Interactions between male-male competition and female mate choice may also have significant consequences for the strength and direction of gene flow when females of one lineage prefer the more dominant males of the other (e.g. Grava *et al.* 2012). Thus, patterns of introgression between lineages, and the consequences for speciation will be the outcome of both female choice *and* male-male competition. It would therefore be interesting to know, for example, whether female choice for divergent male sexual signals and asymmetries in male competitive ability have contrasting or complementary effects on the rate and phenotypic targets of introgression, as would be expected if the former typically promotes assortative mating and the later disassortative mating (although see Otto *et al.* 2008). In relation to this, the direct role of inter- and intra-sexual selection in reproductive isolation or adaptive introgression will, in part, depend on the ability or each lineage to perceive members of the other as mates or competition i.e. whether the sexual signals of each lineage fall within the range of recognition of males and females (Ryan & Rand 1993). To understand the extent to which this is the case, experimental studies linking individual phenotypes and the outcomes of male-male competition and reproductive interactions to reproductive success (e.g. **Chapter 3**) are useful. Furthermore, as demonstrated in this thesis, such approaches should be used to generate predictions regarding the direction of evolutionary change following secondary contact (including whether phenotypes are likely to converge or diverge).

Females may contribute to patterns of introgression in subtle ways in wall lizards. The work in this thesis suggests that asymmetries in the outcome of male-male competition alone may be sufficient to cause asymmetric hybridization and sexually selected introgression in wall lizards. Nonetheless, females may still contribute to patterns of introgression via more subtle

mate preferences, such as the rejection of low ranking males (Parker 1983), or differential reproductive allocation in response to males of higher phenotypic quality (Sheldon 2000). Indeed, female rejection of male courtship and copulation attempts is common in lizards, including in this species (Edsman 1990; although rejection behaviour is often associated with a lack of female receptivity e.g. Olsson & Madsen 1995). However, sexual harassment and forced copulation by males is also common in lizards, particularly in more sexually dimorphic species (e.g. Olsson 1995; Le Galliard *et al.* 2005). Given these two factors, even in the absence of female mate preferences *per se*, female behaviour could nonetheless enhance asymmetries in the direction of hybridization as a by-product of lineage differences in male competitive ability and dominance (e.g. in bite force and male dominance, **Chapter 3**). Specifically, Italian females may more often resist forced copulation attempts from Western European males than Western European females from Italian males. Furthermore, Italian females had greater bite force on average than Western European females (**Chapter 2**) and won more female-female agonistic encounters in the mixed-lineage enclosures (of the 19 female-female agonistic interactions that were observed between the lineages the Western European female retreated in 16 cases). Thus, asymmetries in female competitive ability and the outcome of female resistance to mating attempts could potentially enhance the effects of asymmetries in male-male competition on the rates of hybridization and introgression. Notably, however, there was no evidence for biases in female mate rejection with respect to male lineage from the mating trials conducted in **Chapter 2**. Numerous studies have also demonstrated that parental investment, such as clutch size and egg mass, increases when mates are more highly ornamented (e.g. Simmons 1987; Petrie & Williams 1993; Reyer *et al.* 1999). Therefore, depending on the perception of male traits by Western European females, these effects could also enhance introgression. However, there is little evidence to suggest such responses in the wall lizards, (Uller & While, unpublished data).

Rates of introgression in characters can differ depending on the communication channel. In striking contrast to the extensive introgression of the visual characteristics of the Italian

lineage (dorsal and ventral colouration, and head length) into the Western European lineage similar geographic patterns of variation were not detected in the chemical profiles of male femoral gland secretions (**Chapter 4**). The chemical characteristics of the secretions of Italian males instead showed patterns of introgression more similar to that of neutral microsatellite markers. These differences have an interesting evolutionary consequence in that males found in populations between the limit of neutral introgression and leading-edge of the hybrid zone possess novel combinations of visual and olfactory characters (differing from pure individuals of either parental lineage). Thus, this study highlights that hybridization and introgression can facilitate the emergence of novel trait combinations and hence contribute to phenotypic diversity (Soltis 2013).

The results in **Chapter 4** are consistent with several other studies of hybrid zones which have identified differential patterns of variation among signalling components (e.g. den Hartog *et al.* 2010; Greig & Webster 2013). In these cases, geographic patterns have been attributed to the effects of three factors: the context in which each signal is used (i.e. patterns of selection on the character within the contact zone); the degree or mode of heritability in the signal (e.g. genetic or cultural inheritance, plasticity, genotype-by-environment interactions); and the extent to which receiver responses are learned or inherited, and relatedly, whether or not the production and perception of signals can remain coupled during their transmission across the contact zone. For example, in a contact zone between two species of dove, *Streptopelia vinacea* and *S. capicola*, in Uganda, where there is genetic evidence of asymmetric introgression, territorial vocalizations (often genetically inherited in suboscines) are intermediate in hybrids but morphology and colouration are more similar to *S. vinacea* than *S. capicola* (den Hartog et al. 2007; den Hartog et al. 2008; den Hartog et al. 2010). Large variation in the response of individuals from the contact zone to the territorial vocalizations of pure parents and hybrids is suggestive of responses to vocalizations based on experience rather than genetic predisposition. Thus, this has likely affected the extent to which vocal signals have introgressed (den Hartog *et al.* 2008). In perhaps

the best described example of the differential introgression of sexual signals, a contact zone between two subspecies of fairy wrens, *Malurus melanocephal cruentatus* and *M. m. melanocephalus*, the red plumage of *cruentatus* has introgressed extensively but acoustic signals have not (Baldassarre & Webster 2013; Greig & Webster 2013; Baldassarre *et al.* 2014; Greig *et al.* 2015). In this example, the cultural inheritance of songs combined with a potential benefit for males in the maintenance of acoustic divergence (due to reduced agonistic encounters) has probably inhibited the transmission of *crutatus* song in the direction of genetic introgression across the hybrid zone (Greig *et al.* 2015). In contrast, the extensive introgression of red plumage colour is likely a direct consequence of the mechanism driving hybridization and genetic introgression, female preferences in both subspecies for red plumage in extra-pair mate partners (Baldassarre & Webster 2013).

In wall lizards, differential patterns of selection, heritability, and responses by potential competitors or mates could similarly explain differences in the patterns of variation in visual and olfactory traits. The extensive introgression of Italian visual characters - dorsal colour, ventral blackness and morphology - into the Western European lineage is likely due to their phenotypic and genetic correlation with an overall more competitive male phenotype that is under sexual selection (**Chapter 3**). Nonetheless, these different visual characteristics may still function independently, for example, by signalling different aspects of an individual's condition, or because colouration may be more important as an indicator of sex whereas morphology relates directly to performance (e.g. Meyers *et al.* 2006; Robertson & Rosenblum 2010; Vroonen *et al.* 2013). In contrast to colouration and morphology, which typically have strong genetic components, the lipophilic composition of the femoral gland secretions of lacertid lizards may be highly environmentally dependent. This is supported by evidence from experimental manipulations of diet and basking opportunity (e.g. Kopena *et al.* 2011; Heathcote *et al*. 2014) and the low intra-individual repeatability of male profiles documented in **Chapter 4**. Thus, novel combinations of visual and olfactory traits might arise within the hybrid zone as chemical

signals respond to variation in environmental factors. While environmentally induced chemical variation likely contributes to observed patterns (e.g. as suggested by smoother clinal variation in chemical profiles across the contact zone compard to other traits, **Chapters 2 & 4**), this explanation is unlikely to fully account for the close correspondence in the position of the chemical cline and that of the hybrid index based on neutral microsatellite markers. Importantly, chemical divergence does not appear to have hindered the introgression of other sexual characters or have a strong role in shaping patterns of hybridization or gene flow between the lineages. Moving forward, establishing the genomic basis of male sexual characters and patterns of introgression could address some key outstanding questions relating to the nature of gene flow in this system. For example, are the genomic regions of the traits that showed patterns of adaptive introgression under direct selection (such as dorsal and ventral colour) or are they linked to other regions under direct selection? Is introgression restricted to a small region of the genome or is it extensive, and to what extent is diversifying selection maintaining differences between the lineages? Are there other functionally relevant loci that show evidence for selective introgression (i.e. unexpected phenotypic targets for introgression), and are they associated with sexually selected phenotypes or other fitness-related traits? Advances in genomic techniques coupled with the availability of the genome sequence for *P. muralis* (unpubl. draft version available), will enable these questions to be addressed (also see reviews, Barrett & Hoekstra 2011; Tigano & Friesen 2016). Whole-genome sequences coupled with genome-wide SNP markers have provide detailed information about variation within hybrid zones and the nature of ongoing introgression in other taxa (e.g. Fitzpatrick *et al.* 2010; Teeter *et al.* 2010; Zhang *et al.* 2016). For instance, in a hybrid zone between *Mus musculus* and *M. domesticus,* a nearly 50-fold variation in levels of introgression among SNP markers has been reported with genomic regions associated with olfaction and responses to pheromones showing patterns of extensive and asymmetric introgression (Teeter *et al.* 2010).

Why is there geographic variation in sexual selected characters in wall lizards? One inescapable question resulting from the findings in this thesis is what drove divergence between Italian and Western European lizards in male sexual morphology and behaviour? The most closely related mitochondrial clades to the Italian lizards studied here in the context of hybridization (all of the Tuscan clade) have Western European-like male morphology. Thus, the exaggerated behaviour, morphology, and colouration of the Tuscan clade may have a very recent evolutionary origin deriving from a Western-European-like ancestor (Yang, While & Uller, unpublished data). While assessing the impact of past selective regimes is inherently speculative, investigating the selection regimes and social and ecological factors associated with divergence in male sexual phenotypes among contemporary populations could be informative. Results from this thesis provide some initial insights. Notably, positive and directional selection gradients on dominance and testes mass, and negative directional selection on outer ventral scale UV hue in males of the Italian lineage (based on the enclosure experiments) are consistent with the direction of shifts in these traits that have occurred between the two lineages. Together with evidence for a stronger Bateman gradient in the Italian lineage, this is suggestive of ongoing patterns of sexual selection on Italian males. Thus, this supports a hypothesis whereby allopatric divergence between the Italian and Western European lineages has occurred via sexual selection.

However, this still does not address the question of what drove initial shifts in sexual selection. Shifts in sexual selection regimes generally require ecological change. For example, via changes in population density, which affects the rate at which individuals meet potential mates and competitors (Kokko & Rankin 2006) or changes in interspecific competition over shared ecological resources (Grether *et al.* 2013). There is little evidence for variation in population densities between the Italian and Western European lineages in their contemporary ranges. However, higher population densities in southern compared to northern glacial refugia during the Pleistocene could be a historically contributing factor to the intensification of sexual

selection on Italian males and phenotypic divergence. Furthermore, intensified competition for basking sites and territory has the potential to drive rapid phenotypic change in males and females through natural selection (e.g. Calsbeek & Cox 2010). Alternatively, the current geographic distribution of the Italian lineage overlaps with several lacertid species of similar size and ecology (Kwet 2009), creating the potential for selection through inter-species competition. For example, both sexes of the Italian lineage have diverged from the Western European lineage in dorsal body colouration, which could represent convergence in signalling systems with sympatric species such as *Podarcis siculus* (Rafinesque 1810). These two hypotheses could be tested using natural environmental variation among extant wall lizard populations. In particular, insular populations of *P. muralis,* such as those occurring in the Tuscan Archipelago, are often characterized by high inter-island phenotypic variability including in the degree of sexual dimorphism (e.g. Bellati *et al.* 2011; Sacchi *et al.* 2015). Population density as well as the number and density of interspecific competitors also vary among islands across the wall lizard range. Thus, they offer natural tests of phenotypic responses to environments characterized by high and low population density as well as high and low interspecific competition, without the confounding effects of hybridization.

6.2 Responses in female reproductive investment and male sexual strategies in a more seasonal environment

Populations can respond rapidly and adaptively to novel climatic conditions. Female reproductive characters (clutch size and clutch mass) differed adaptively between native populations in Italy, where the climate allows for the recruitment of up to three clutches, and non-native populations in England, where the shorter breeding season and cooler climate strongly limits the survival prospects of second and third clutches (demonstrated by While *et al.* 2015). This finding is consistent with patterns of female reproductive investment in association with temperature gradients in other species of lizards (e.g. Angilletta *et al.* 2004; Roitberg *et al.* 2013; Du *et al.* 2014). Environmental temperature could indirectly bring about greater first clutch investment by non-native compared to native females. For example, in the eastern fence lizard, *Sceloporus undulatus*, structural equation modelling has been put to use to evaluate the role of direct and indirect effects of climate on reproductive investment (Angilletta et al. 2006). Cooler climate causes reduced growth rates and increased age at first reproduction (hence delayed reproduction until reaching a larger body size), which allows greater capacity for energy storage by females. This in turn facilitates the production of more eggs, which are favoured by fecundity selection on the adult, and larger eggs, which are favoured for offspring survival. This is consistent with the effects reported in this thesis. It is unclear whether genetic changes contribute to the differences between native and non-native females. Common garden experiments carried out under both native and non-native climatic conditions would help to establish if the divergence in female investment strategies in non-native wall lizard populations represents genetic adaptation or phenotypic plasticity in response to the non-native environment. Nevertheless, the within-season investment strategies of non-native females clearly occur in the direction favoured by selection and suggest a progressive reduction in investment in second clutches.

Data on relative investment by native and non-native females in third clutches could provide further insights into reproductive responses in non-native populations. Genetic variation for the production of two rather than three clutches compared to one clutch rather than two clutches is likely to be more common in the native range in Italy (since at least two clutches will almost always be successful). Hence, it may be easier for non-native populations to evolve a response to a cool climate that completely eliminates the third episode of reproduction. Indeed, the ability to produce three clutches may be largely absent in non-native females. It would also be interesting to monitor the frequency of the production of second clutches by females in non-native populations since genetic variants of females that reproduce only once per breeding season should be favoured. Furthermore, if this female strategy spreads to high levels within populations, under the hypothesis that receptive females are the cues to which males respond, non-native males should then begin to 'switch off' their sexual behaviour later in breeding season.

***Non-native males appear not to alter their sexual* strategies.** Despite the seasonal constraint on the timing of reproductive fitness in England, behavioural observations and measurements of variance in fertilization success taken from native and non-native experimental populations indicated that male-male competition over the second clutches was as intense for non-native as for native males. Thus, the data in **Chapter 5** suggested that introduced male wall lizards may be lagging behind females in their reproductive responses to a new climatic selection regime. However, unless the costs to males in some way limit female fecundity, the slower adaptative responses in males will not necessarily have detrimental consequences for the viability or growth of non-native populations in England. At a mechanistic level, it would also be interesting to investigate the testosterone profiles of native and non-native male wall lizards across the breeding season to see if this corroborates the behavioural data. Neuroendocrine mechanisms underlie male reproductive tactics in lizards (e.g. Knapp *et al.* 2003), thus hormonal responses are expected to be critical in the mediation of adaptive shifts in male sexual behaviour and

traits, and life-history under a new climate (e.g. Atwell *et al.* 2014). More generally, when male sexual behaviour lacks flexibility, a sex-specific pattern in rates of adaptive response (i.e. females first – males lagging) to changes to the environmental context of reproduction may be common. However, since few studies have investigated responses in both female life-history and male sexual strategies under a new climate it is difficult to predict whether or not the effects reported in this thesis will be widespread.

6.3 Concluding remarks

Sexual selection is a common cause of geographic variation in animals. In this thesis, I have experimentally established that divergence in male morphology and behaviour in allopatry promotes asymmetric introgression following secondary contact between two divergent lineages of wall lizard. This work revealed the causes of asymmetric hybridization and suggests that, although visual characters are under sexual selection, femoral secretions appear to be largely selectively neutral in secondary contact. I also provide evidence of sex-specific responses following introduction to a new climatic regime, specifically, a lag in adaptive responses in the reproductive strategies of males. Both investigations support the growing account of the complex ways in which the social and ecological environment can influence the evolutionary dynamics of sexual characters and highlight two environmental contexts where competition among males may have a fundamental role in shaping the direction and tempo of evolution.

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Appendix I

Heathcote, R. J. P., While, G. M., MacGregor, H. E. A., Sciberras, J. , Leroy, C. , D'Ettorre, P., Uller, T., 2016, Male behaviour drives assortative reproduction during the initial stage of secondary contact. Journal of evolutionary biology, 29(5), 1003-1015.